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Assessing the genetic diversity of the genus *Viburnum* using simple sequence repeats

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To the Graduate Council:

I am submitting herewith a dissertation written by Deborah Dean entitled "Assessing the genetic diversity of the genus *Viburnum* using simple sequence repeats." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

Robert N. Trigiano, Major Professor

We have read this dissertation and recommend its acceptance:

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Assessing the genetic diversity of the genus *Viburnum* using simple sequence repeats

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Degree

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Dedication

I dedicate this work to my dear Pippin. You are at once my daughter, my best friend, my constant companion, my protector, and my source of joy and laughter. Although you are gone from my life, you remain in my heart. Pippy you are missed and loved greatly.

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Abstract

The genus *Viburnum* was established in 1753 by Linnaeus and is the largest genus in the Adoxaceae and consists of approximately 160 species. *Viburnum* species are small trees which grow throughout the temperate regions of the Northern Hemisphere (Hoch, 1995). While this genus shares little variety in fruit and flower morphology, it is diverse in many other traits. As molecular studies advance, this large genus continues to undergo reclassification. Here three SSR libraries were constructed to discern additional molecular insight into this vast genus.

Microsatellite markers were developed to characterize *Viburnum* on several different levels. *V. dilatatum* is an introduced Asian species that has a wide range of desirable horticultural traits, but this is countered with a propensity for its seedlings to become invasive, therefore creating an interest in the genetics and breeding of this species. Eleven SSR markers were used to characterize *V. dilatatum*, and are expected to aid in breeding programs that are attempting to develop new cultivars and assist with early detection and screening of plants that have escaped cultivation.

Genetic diversity and population structure was examined in the native species *V. rufidulum*. The populations examined were found to have a low to moderate genetic differentiation and high level of gene flow. The greatest genetic variation was found to exist within populations. This coupled with the high cross transferability of the SSR markers to other *Viburnum* species are expected to be helpful in refining the phylogenetic relationship of *Viburnum* and other genera in Adoxaceae.

In the past the *Viburnum* has undergone various reclassifications based on morphological and molecular studies. Thirty-three microsatellite markers were developed from *V. dilatatum, V. farreri*, and *V. rufidulum* and utilized to characterize the relationships between various taxa.

Cross transferability of the loci was analyzed and the polymorphic information content ranged from 0.42 to 0.97%. Three loci were used to construct a phylogenic tree. Sequence alignments indicated well preserved primer sites and resulted in cross transferability among many *Viburnum* species and to other genera. We expect this set of SSR markers to of utility in future genetic studies, marker assisted selection, and breeding programs.

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Chapter 1. Introduction

Assessing the genetic diversity of *Viburnum* species with simple sequence repeats

Viburnum is the largest genus in the Adoxaceae with approximately 158-200 species (Winkworth and Donoghue, 2005; Jacobs et al., 2008) and was formerly a member of the honeysuckle family (Caprifoliaceae). *Viburnum* species are a diverse group of shrubs and small trees found growing throughout the Northern Hemisphere in temperate and subtropical regions (Hoch, 1995). There are approximately 20 North American species, 60 species from South and Central America, four European species, 30 North African species and more than half of all species are indigenous to Asia (Dirr, 2007).

In North America, *Viburnum* is valued as a hardy and versatile plant that provides year-round color to landscapes. *Viburnum* has showy white or pink flowers (with some rivaling the beauty of hydrangea) that contrast green glossy leaves in the warm months, and then gives way to a spectacular show of color in autumn as foliage turns to hues of yellow, red and gold. The fruits are attractive occurring in various shades of blue, purple, black, red, orange and yellow, whereas some have metallic tones. The multiseasonal allure makes Viburnum popular and as a result, it is an important crop in the nursery industry, accounting for approximately $19,500,000 in retail and wholesale sales (USDA, 1998). *Viburnum* are an ecologically important group of plants, and received a “moderate” rating from the USDA Plants data base, because of its importance to local wildlife as a provider of shelter and food. Fifteen indigenous or naturalized species are categorized as endangered.

In spite of popularity, ubiquitous nature, and economic value, absolute classification of *Viburnum* remains elusive despite many attempts to organize this large genus. Due to worldwide distribution, there are many wild and horticultural hybrids, making taxonomical determination a

challenge (Hoch, 1995). There is no consensus of the taxonomic importance of morphological differences but Dirr (2007) stated that the only traits common to all *Viburnum* species are fruit (drupe) and leaf morphology (opposite). However, Donoghue (2004) indicated that while flower and fruit characteristics are conserved, there is also great variation within the genus. These same authors noted that while fruit color, leaf shape, inflorescence, and bud morphology differ greatly, these attributes have been used to classify the genus. This lack of agreement compounds the complexity of classification in *Viburnum*, and indicates a need for a better classification system.

The genus is valued in Asia as a Chinese popular folk medicine and has been used to treat diarrhea, rheumatoid arthritis and cough (Wang, L et al., 2008). *Viburnum dilatatum* has recently been cited in studies evaluating phenolic antioxidant levels in plants (Iwai et al., 2004; Wang et al., 2008). The fruit of *V. dilatatum* contains compounds that have high antioxidant activity, which may ameliorate oxidative damage (the precursor of some cancers and disease) caused by stress (Iwai et al., 2001). This untapped resource could make *Viburnum* increasingly valuable in the future. Other species in the genus could also contain the compounds that are medicinally important, but further studies are needed (Wang, L et al., 2008). Establishing genetic diversity could assist in medicinal use of *Viburnum* by identifying other species closely related to those currently being used. This study will provide that initial genetic information.

Viburnum is an environmentally hardy plant, but is susceptible to infestation by *Pyrrhalta viburni*, the viburnum leaf beetle in northern areas of the U.S. (P.Weston, personal communication). The beetle, first introduced into Canada from Europe, has spread rapidly towards the south in the U.S, and is expected to become widespread because its native range includes all of continental Europe (Weston and Desurmont, 2002). Interestingly, Asian species of *Viburnum* appear to have resistance to the beetle and various North American species range from

resistant to highly susceptible (Weston and Desurmont, 2002). American species such as *V. trilobum* and certain hybrids are highly susceptible to infestation (Weston and Desurmont, 2002). Conformation of those North American species more closely related to the Asian plants will assist in beginning to examine if the Asian group can confer resistance to others. Knowledge of susceptibility can help breeders incorporate resistant plant material into their germplasm (Weston and Desurmont, 2002). The first set of SSR markers developed for this study were created from the Asian species *V. dilatatum,* and could prove useful in a breeding program to make crosses that are increasingly resistant to the leaf beetle, since Asian species carry resistance to Viburnum leaf beetle. Microsatellites will also assist in correlating heredity and resistance between the myriad *Viburnum* hybrids.

OVERVIEW OF PREVIOUS STUDIES

Previous studies of *Viburnum* concentrated efforts on using various genes or combinations of genes in an attempt to organize and resolve relationships within this large genus. As reported by Winkworth and Donoghue (2005), Linnaeus organized the genus in 1753, and in 1861, Oersted identified the main characteristics of the group. Rehder (1940) delineated nine sections with a concentration on Asian species, and later Hara (1983) described sub-generic names and added another section to the nine previously recognized sections. With these exceptions and a few other regional treatments, mostly with descriptions and keys, there have been few sectional revisions (Winkworth and Donoghue, 2005). Nuclear (granule-bound starch synthase genes and nrITS, chloroplast (*trnK* intron and *psbA-trnH* intergenic spacer, genes were used to examine the phylogeny of the genus (Winkworth and Donoghue, 2004; Winkworth and Donoghue, 2005). The 2005 study suggested 12 clades where plants could be placed until future studies included or excluded individuals from specific clades (Winkworth and Donoghue, 2005).

The results of their molecular work helped clarify relationships lineages of the sections; however the ancestral groups at the phylogeny remain nebulous (Winkworth and Donoghue, 2005).

Jacobs et al. (2008) expanded the work of Winkworth and Donoghue (2005) and examined the morphological characteristics of endocarp and seed, using the fruit as a classification tool. The 2008 study found evidence in pyrene shape and grooving to support both Hara (1983), and Winkworth and Donoghue's (2005) division of the genus, but found further clarification of phylogeny, and broader sampling necessary to understand characteristic development within the various clades. The genus is ancient and fossil remains have been recorded from various regions of the world. It is thought that the plants differentiated long ago, evolving to create myriad variations in fruit, flower, leaf, winter bud, and habit (Hara, 1983).

In 2009 the Plant Working Group of the Consortium for Barcodes of Life suggested that the chloroplast coding genes *matK* and *rbcL* be used as a standard for DNA barcoding and hence identification of plants (CPWG, 2009). Clement and Donoghue (2012) followed the recommendations of the Plant Working Group of the Consortium for Barcodes of Life to further compare relationships across and within *Viburnum*. The outcome of that study found, although the *matK* and *rbcL* loci were capable of distinguishing species across the various clades, there was a high rate of failure and low levels of discrimination in closely related species of *Viburnum.* The authors went on to suggest that those loci could reduce the success in phylogenetic studies of other genera of woody plants as well. Furthermore they urged those involved with plant barcoding to increase the number of loci utilized in order to discriminate closely related species (Clement and Donoghue, 2012).

Additionally, NCBI GenBank, the repository for available DNA sequences, does have molecular sequence data of some chloroplast genes for *Viburnum* (Winkworth and Donoghue,

2005), however, there are limited SSR sequences. There is however, agreement that further molecular studies will be of value to resolve remaining issues of relatedness within the 12 sections and at the base of the genus (Jacobs et al., 2008; Winkworth and Donoghue, 2005). Molecular markers are ideal tools to study relatedness of species and assist in mapping valuable traits; this study will employ the SSR markers.

SSRs

Molecular tools currently being used to study genetic diversity include random amplified polymorphism (RAPDs), amplified fragment length polymorphism (AFLPs), restriction fragment length polymorphism (RFLPs) and simple sequence repeats (SSR)s- also referred to as microsatellites. SSRs have proven more valuable in determining genetic diversity and parentage because they are ubiquitous throughout plant genomes, are highly polymorphic, and are codominant (Pejic et al., 1998). When RFLPs and SSRs were compared, 15% of RFLP probes were able to detect three or more alleles, as opposed to 80% found by the use of the SSRs (Powell et al., 1996). Initial development of SSRs is expensive and labor intensive, but once developed, this approach becomes cost effective (Mace and Godwin, 2002).

Microsatellites are tandemly repeating mono-, di-, tri-, tetra, or penta-nucleotide segments of DNA that are situated throughout the genomes of most eukaryotic species (Powell et al., 1996). SSRs are polymorphic, ubiquitous throughout plant genomes, sexually transmitted codominately (establishment of hybridity), and require relatively small amounts of DNA to develop (Powel et al., 1996). Microsatellites also provide a means of standardizing research findings because the information can be shared between scientists in the form of primer sequences (Silver, 1992). Scientists are therefore able to build on, and expand previous studies. It is possible to extract DNA, and isolate SSRs so that genetic information may be extrapolated.

Standard methods to obtain microsatellites include the following: creation of a small insert genomic library, library screening via hybridization, SSR positive clones are sequenced, primers designed, and polymorphisms identified (Powell et al., 1996). The regions flanking the SSR loci are also valuable in genetic studies. Conservation of the SSR unique flanking region allows for the design of primers that will amplify the microsatellite loci during PCR (Mace and Godwin, 2002). Polymorphisms of the microsatellites loci are alternate forms of a gene, and can be detected easily via PCR (Powell et al., 1996; Zhao et al., 2008). Parentage can be established or confirmed with SSRs because parents confer allelic composition to their offspring at the same loci (Yamamoto et al., 2003). Wadl et al., (2008) examined trees in a dogwood breeding line and demonstrated how SSRs assist in determining identity of similar cultivars, establish genetic diversity, and resolve parentage of species. SSRs have also been utilized to locate disease-resistant genes (Wang, X. et al., 2008). In another study, SSR loci that amplified across Hydrangea species were used to create a marker-assisted breeding program (Rinehart et al., 2006). Having a basic knowledge of the parents' genetic makeup can help save plant breeders' money, resources and precious time. Clarification of genetic similarities and differences provides valuable information when choosing parents for hybrid development and accelerates breeding programs (Rinehart et al., 2006). Powell et al. (1996) stated that the potential use as a diagnostic marker in breeding programs is one of the main reasons to develop SSRs. *Eucalyptus* spp. SSRs amplified to *Acca sellowiana* demonstrated that microsatellites will transfer to plants within another closely related genus (Louise dos Santos et al., 2007). Therefore, applying SSR markers to *Viburnum's* nearest relative, *Sambucus,* may resolve the genetic confusion at the base of the 12 established sections. Because SSRs are able to exploit genetic heritage and can be employed across different genera they were utilized in this study. As previously stated, most of the

molecular studies of *Viburnum* to date, have involved markers other than SSRs, which presented an ideal opportunity for this study, and building DNA libraries is a practical way to find SSRs (Hamilton et al., 1999).

OBJECTIVES

The first objective of this thesis is to develop a molecular tool, in this case microsatellites, from two or three SSR-enriched *Viburnum* species genomic libraries. *Viburnum dilatatum, V. farreri* and *V. rufidulum* will be used because they are well separated within the phylogeny supported by Winkworth and Donoghue (2005). SSRs will assist in further population genetic studies of these plants. Microsatellites will be isolated and primers designed for the three separate species, and will provide broad representation of the genetic range of the genus. The second objective is to assess the ability of selected SSRs to amplify across various *Viburnum* species. This will evaluate genetic diversity and similarities through the degree of conservation of the microsatellites. The third objective is to collect approximately 20 to 25 accessions of one of the native species, *V. rufidulum*, found growing locally, and use microsatellites to evaluate the genetic diversity. Self-incompatibility is widespread within the genus (Hoch et al., 1995). However, it has been observed that in *V. dilatatum* and *V. dentatum,* plants isolated from other species will set fruit (Dirr, 2007). If the plants are in fact self-sterile, some the species could actually be hybrids. Applying SSR markers to a native population will provide genetic fingerprints with which to compare that population.

Material and methods

Plant Material

Unopened buds and young leaves from 44 *Viburnum* species and cultivars (Table 2) were collected and stored in ethanol at 20°C. Accessions were obtained from Professor Tom Ranney, Department of Horticultural Science, North Carolina State University, Raleigh, NC.

Isolation and digestion of genomic DNA

Genomic DNA from three species (Table 1) of Viburnum will be used to construct libraries enriched with the dinucleotide repeat GT/CA. GT motifs were found to be the most abundant dinucleotide (42%) in a previous study (Mace and Godwin, 2002). GT enriched genomic libraries will be created for the following species: *V. dilatatum*, *V. farreri,* and *V. rufidulum*, which represent both Asian and North American species. DNA from *Viburnum dilatatum* 'Asian Beauty' will be used to build the first genomic library. Genomic DNA from 'Asian Beauty' was extracted from previously collected buds and leaves and stored at –80 °C using a DNeasy ® Plant Mini Kit 250 (Qiagen, Germantown, MD, USA). Isolated DNA was quantified using the NanoDrop® ND-1000 ultraviolet-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Quality was established utilizing 2% agarose, ethdium bromide stained gels and visualized in a 2000 Gel Doc System (Bio-Rad Laboratories, Hercules, CA, USA).

Construction of the enriched genomic libraries will be based on methods described by Wang et al. (2007). Approximately 20 to 30 mg of leaf or bud tissue will be ground and DNA isolated using the Qiagen Dneasy Kit. Following isolation, genomic DNA (≈ 2.5 µg) will be digested for 3 min at 37°C using each of the following restriction enzymes: *Alu I, Hae III, Rsa I* and *Stu I*-0.5 µl 10U/µl, each in a solution including NEB Buffer, BSA, and water for a total 100 µl. reaction mixture, which yields blunt ended fragments approximately 200-800 base pairs (bp)s

in length. The size is important because fragments must fit within the T7 and T3 restriction sites within the *Eco*R V- pBluescript SK II (+) (Stratagene) plasmid/vector, which requires that DNA fragments be less than 1000 bp (Hamilton, et al., 1999). The restriction enzymes will be heat inactivated at 70°C for 20 min. Blunt end fragments will be repaired using the End-it® DNA End-Repair Kit (Epicentre, Madison, WI), and purified digested genomic DNA will undergo dephosphorylation of the 5' ends and be ligated to SNX linker adapters, which will be adapted to GT biotinylated oligonucleotides (Hamilton, et al., 1999). Electrophoresis of PCR products will confirm successful linkage to genomic DNA ligation, which will present as a smear longer than that of the PCR products of genomic DNA (Numone et al., 2006). A 100-µl solution combining 48 µl of linker ligated fragments, 2 µl of 5' biotinylated repeat oligonucleotide $GT_{(12)}$ 1 µM, and 50 ul of 2× hybridization buffer (12× SSC/0.1% SDS) with a drop of mineral oil (to prevent evaporation) will be prepared. The sample will be denatured at 95°C for 15 min and the hybrids bound to the streptavidin-coated beads (Streptavidin Magnesphere® Paramagnetic Particles -Promega, Madison, WI, USA) at 43°C for 30 to 90 min, then placed on a magnetic stand for1 min and washed twice with 200 µl of 2× SSC/0.1% SDS for 5 min at room temperature. The hybrid bound streptavidin-coated paramagnetic particles will be washed twice for 5 min at 45°C with 200 µl of 1× SSC, 0.1%SDS, and then washed twice at 65°C with 1X SSC, 0.1% SDS (using 100 µl 1× SSC, 0.1% SDS for the second wash). Each wash will be followed by placement on the magnetic stand for 1 min to retain the motif containing fragments, and remove any genomic DNA not bound to the streptavidin-coated paramagnetic particles. The captured fragments will be eluted with 60 µl sterile water and heated with the 125 D Isotemp (Fisher Scientic, Waltham, Massachusetts, USA) to 95°C for 15 min, and then quickly separated on the magnetic stand, a process that releases the bound genomic DNA from the paramagnetic

particles (Hamilton et al., 1999). The enriched fragments will be purified with the QIAquick® PCR purification Kit (250) (Qiagen, Valencia, CA) to eliminate lingering biotinylated oligonucleotides and buffers. Repeat enriched genomic DNA will be amplified in a reaction mixture of 10 µl post hybridized DNA, 5 µl 10X Thermopol Buffer, 5 µl 2 mM dNTP, 4 µl 2 µM SNX forward linker, 0.3 µl 2U/µl $Vent_R$® (exo-) polymerase and 25.7 µl PCR quality water for a total solution of 50 µl at PCR settings of 96°C for 45 s, 62°C for 1 min, and 72°C for 2 min for 40 cycles. To encourage blunt ends *Stu* I (1 µl) will be added to the 50 µl of PCR product and incubated for 6 h at 37°C for digestion, the restriction enzyme will be inactivated by dropping the temperature to 70°C for 15 min. Blunting of enriched DNA ends will facilitate ligation with the plasmid E*co*R V pBluescript SK II (+) for subsequent transformation into *Escherichia coli* electrocompetent cell TOP 10 Electrocomp™ cells (Invitrogen, Carlsbad, CA, USA).

Purified post SSR enriched fragments will be ligated to the *Eco*R V digested pBluescript SK II (+) vector DNA using a fragment to vector ratio of 1:1 with the following PCR reaction: 0.5 µl 10 ng of cleaned post enriched DNA, 2 µl 10X Ligase Buffer, 0.2 µl 100X BSA (bovine serum albumin), 1 µl 14 µg EcoR V-digested pBluescript SK (+) plasmid, 0.5 µl 2000 U/L ligase, and 15.3 µl PCR ready water. The reaction will be placed in the thermocycler overnight at 30 min cycles at 16°C. Dialysis to remove unwanted salts and buffers will be performed by placing 20 µl of vector-ligated genomic DNA on an MC-free filter placed in water until a final volume of 12 µl is reached.

The vector ligated genomic DNA will be transformed into *Escherichia coli* electrocompetent cell TOP 10 Electrocomp™ Cells (Invitrogen, Carlsbad, CA, USA) with the Gene Pulser Xcell® Electroporation System (Bio-Rad, Hercules, CA) using Voltage 2500, Capacitance, uF 25, and Resistance 200. To facilitate recovery of cells, putatively transformed

cells will be grown in Super Optimal Broth medium without antibiotics and incubated at 37°C for 40 min with continuous shaking at 200 rpm. The bacterial cells will be plated on LB-amp$_{100}$ solid medium petri plates containing IPTG and X-gal and incubated overnight at 37°C for color expression and cell growth (Sambrook et al., 1989).

The bacterial cells that have been successfully transformed will survive because the plasmid pBluescript SK II (+) will confer ampicillin resistance (Clark and Russell, 2005). pBluescript SK II (+) also carries the *lac*Z gene that codes for β-galactosidase, which will turn X-gal blue (Clark and Russell, 2005). When the transformed bacterial cells are exposed to X-gal on the plates, colonies will turn blue indicating the transmission of the plasmid/vector alone (Chaffin and Rubens, 1998). The desired white color occurs when the plant DNA fragment has been inserted into the multiple cloning site (MCS) of the plasmid; interrupting the Lac Z gene which codes for the typical blue color, thereby disrupting the gene function, so the cells remain white. The desired white colonies will be transferred to 96-well plates containing a mixture of 200 µl LB freezing medium and 100 µg/ml ampicillin (Sambrook et al., 1989). The plates were incubated at 37°C overnight and then screened for GT motif containing colonies by PCR. Individual white colonies will be selected using a sterile tooth pick and the following reaction mixture was inoculated with the putative colony. The PCR reaction will consist of a 10 µl total volume that included: 1 µl 2 mM dNTP, 1 µl 10× PCR gold Buffer, 1 µl $MgCl_2$, 1 µl T7 2.5 mM primer, 1 µl T3 (2.3 nM) primer, 1 µl GT_{12} 2.5 mM, respectively, 0.07 µl AmpliTaq Gold with Geneamp (manufactured by Roche) Taq polymerase, 4 µl water, and 10 µl of the mixture will be placed in each 96-well of the plate. The 96-well plate will be PCR amplified using the Eppendorf Mastercycler thermocycler (Hamburg, Germany) at 96°C for 2 min, 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for a total of 40 cycles. Following amplification, products are to be

electrophoresed on a 2% agarose gel for 40 min. The clones will be considered positive for containing a SSR motif if a smear pattern is observed when PCR products are visualized with ethidium bromide (Wang, X. *et al.,* 2007). Furthermore, the smears will be compared to the 100-bp ladder for fragment size estimation, and samples that show greatest "intensity" at the 500 to 800-bp range of the ladder, will indicate ideal fragment size. Of the 768 smear-positive and white colonies examined thus far, two 96-well plates were sent to the USDA Genomics and Bioinformatics Research Unit in Stoneville, MS to be sequenced. Primer pairs have been developed with primer 3.0 (Whitehead Institute of Biochemical Research, Cambridge, MA) (Wang, X. et al., 2007; Wadl it al., 2008). Of the 192 colonies sent to the USDA for sequencing, 47 primers (forward and reverse pairs) were designed to complement regions flanking the genomic DNA microsatellites. Primers have been evaluated on the basis of adequate flanking region size and presence of dinucleotide motifs. Each of the 47 primers have been screened against genomic DNA of the *V. dilatatum* cultivars, Asian Beauty and Erie. A 10-µl PCR solution consisting of 0.2 ng of genomic DNA, 1 µl 2 mM dNTP, 1 µl 10× PCR gold buffer, $MgCl_2$, 0.08 µl U AmpliTaq Gold® has been run on an Eppendorf Thermocycler at 94°C for 3 min., 94°C for 40 s, 55°C for 40 s, 72°C for 30s for 35 cycles and 72°C for 4 min with a final extension of 72°C for 4 min. The products have been separated on 2% agarose gel and visualized with ethidium bromide. I have completed this library and of the 47 primer pairs tested, thirty (64%) produced PCR products indicating successful optimization of the primers; 23 generated polymorphic loci between *'*Asian Beauty' and *'*Erie*'*; and four loci were monomorphic.

Currently the *V. farreri* and *V. rufidulum* genomic libraries are being constructed. Permission to collect 25 native *V. rufidulum* specimens have been obtained from the Tennessee

wildlife resource agency and those plants will be collected from the Forks of the River area by the end of February 2009. Work continues to optimize the remainder of *V. dilatatum* primers.

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Appendix 1: Tables

Table 1-1. *Viburnum* species used to create genomic libraries in this study

Viburnum used to create genomic libraries in this study to include taxa, origin and clade.		
Taxa	Origin	Clade
V. dilatatum	Korea, China, Japan	Succodontotinus
V. farreri	China	Solenotinus
V. rufidulum	southeastern United States	Lentago

Chapter 2. Screening and characterization of eleven novel microsatellite markers from *Viburnum dilatatum*.

This chapter is a revised version of a submitted manuscript of the same name that appeared in the journal HortScience in 2011. The authors are Deborah Dean, Phillip Wadl, William Klingeman, Bonnie Ownley, Timothy Rinehart and Robert Trigiano.

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My principal contribution are (1) project selection and development; (2) review of literature pertinent to this project; (3) analyzing and summarizing all data; (4) editing and synthesizing the contributions of the co-authors into a single paper, and (5) the majority of writing of this work.

Abstract

Viburnum dilatatum is a popular and economically important ornamental shrub. The wide range of desirable horticultural traits, paired with a propensity for seedlings to become invasive, have created interest in the genetics and breeding of this species. To investigate the genetic diversity of *V. dilatatum*, microsatellite loci were identified from a GT- enriched genomic library constructed from V. dilatatum 'Asian Beauty.' Eleven microsatellite loci have been characterized on a group of sixteen different related *V. dilatatum* cultivars and hybrids. Two- to- twelve alleles were identified per locus, and the polymorphism information content (PIC) values ranged from 0.36 to 0.87. Expected heterozygosity (He) [expected heterozygosity] ranged from 0.48 to 0.88 and observed heterozygosity (Ho) [observed heterozygosity] ranged from 0 to 0.73. This set of molecular markers also exhibited expected transferability between various *V. dilatatum* cultivars and two hybrids with *V. japonicum*. As a consequence, these markers will aid in breeding for new cultivar development, assist with early detection and screening of plants that have escaped cultivation, and are expected to help in refining the phylogenetic relationship of *V. dilatatum* to other species and genera within the Adoxaceae.

Introduction

The genus *Viburnum* includes more than 160 species of shrubs and small trees distributed widely throughout the Northern hemisphere and into the Southern hemisphere (Winkworth and Donoghue, 2004). Collectively, *Viburnum* species provide many ideal year-round ornamental qualities. The attractive flowers and glossy green leaves of spring and summer give way to an array of bright berries and vibrant fall foliage with colors ranging from yellow to red to dark purple. The multi-seasonal allure of plants in this genus makes Viburnum an important ornamental plant crop to the United States (U.S.) nursery industry. Total sales of *Viburnum* were

$19,500,000 in 1998 (USDA, 1998), and sales increased to $24,647,000 in 2007 (USDA-NASS, 2010). Growing awareness and ongoing breeding efforts have led to increasing consumer demand for viburnum shrubs. *Viburnum dilatatum*, a common and versatile species that is native to Asia, was introduced to the U.S. in 1846 and is valued for its vibrant fall color, leaf color retention, and outstanding cherry-red fruit production that persist into winter. For these reasons, it has become increasingly prevalent in horticultural commerce (Dirr, 2007). At maturity, the shrub reaches 1.5 to 3.0 m (5- to -10 feet) tall and exhibits lustrous dark green leaves.

There are striking diversity among horticultural characteristics in *Viburnum* species, including leaf shape, fruit and flower color, fertility, and plant growth habit (Dirr, 2007; Winkworth and Donoghue, 2004). Erected by Linnaeus in 1753, the genus has undergone more than ten taxonomic revisions, and in spite of those classification efforts, evolutionary relationships and geographical relatedness of the species have been largely unaddressed (Winkworth and Donoghue, 2005). Winkworth and Donoghue (2005) combined molecular data utilizing two chloroplast genes (trnK intron and psbA-trnH IGS) and three nuclear loci (nrIts, WAXY1 and WAXY2) to provide a biogeographical analysis of the genus. Although twelve well-supported species groups, or clades, were identified using the molecular data, issues within this eclectic genus still linger. For example, the relationship between the section Pseudotinus and the species *V. clemensiae* and *V. urceolatum* is not well supported. In addition, the rates of diversification remain unclear, and there is a persistent lack of resolution at the base of the phylogenetic tree (Winkworth and Donoghue, 2004; Winkworth and Donoghue, 2005). Not surprisingly, a plethora of cultivars and hybrids further complicate the organization of this vast genus (Dirr, 2007).

While many ornamental plants, including *V. dilatatum*, make important contributions to regional environmental health and diversity, certain non-native species have escaped cultivation and are causing economic damage. During a period of 85 years (1906-1991), 79 exotic plant species have been deemed responsible for almost $97 billion in damages in the U.S. (OTA, 1993). Many of the plants listed on invasive species lists, such as *Celastrus orbiculatus, Euonymus alatus*, and *Pyrus calleryana*, remain economically important and popular nursery plants (University of Georgia, 2010). Regardless, invasive plants cause significant economic damage, and can lead to considerable reductions in populations of native flora and fauna, often in response to competition for limited resources (Anonymous, 2008). Unfortunately, current methods of non-native plant management, which include physical removal, and herbicidal and biological control methods, have not been practical or effective in controlling extensive proliferation of many invasive ornamental plants (Li et al., 2004). Indeed, the increase in commercial availability of potentially problematic plants makes early characterization of potential pest plants and their cultivars a high priority. The New Jersey Invasive Species Strike Team placed *V. dilatatum* on a 2010 Watch List of invasive species that identifies plants that may pose a threat to native plant communities (Anonymous, 2010). *Viburnum dilatatum* can form thickets after escaping cultivation that may displace or prevent growth of less competitive native herbs, shrubs, and trees. Shrubs yield an abundance of viable seed from a typically heavy seasonal fruit set (Dirr, 2007). In turn, viable seeds aid in the rapid colonization of land previously not colonized with *V. dilatatum.*

Some invasive species, including *V. dilatatum*, may be difficult to discern from native vegetation (Anonymous, 2008). Dirr (2007) stated that *V. dilatatum* is difficult to identify based on foliage, as some specimens may have branches with two differently shaped leaves co-mingling. Indeed, *V. dilatatum* has been observed along the woodland edges of the National Arboretum, often

growing undetected until old enough to display their unmistakable red fruits (J. Feely, personal communication). Moreover, there is anecdotal evidence to suggest that *V. dilatatum* may be escaping cultivation and hybridizing with the natively occurring *V. dentatum* (J. Feely, personal communication). This caveat necessitates the availability of a reliable method for identification of this popular ornamental shrub and its hybrids.

A group identified as the Invasive Plant Research and Partnerships with Ornamental Horticulture and Natural Resource Management, convened for a workshop in 2008 to discuss the problem of invasive plant species. Research areas were identified and included the following priorities: develop scientific means to evaluate the persistence and spread of invasive plants; enhance detection methods for invasive taxa; acquire a means to identify invasive species, cultivars and hybrids of cultivars, such as genetic markers (Anonymous, 2008). Molecular markers provide a means of meeting these criteria.

Molecular tools have become a superior method for interspecific and intraspecific hybrid analysis and cultivar identification of ornamental plants (Pounders et al., 2007; Wadl et al., 2008; Wadl et al., 2009; Wang et al., 2010). Microsatellites are ubiquitous throughout genomes and codominant markers, often used in both interspecific and intraspecific diversity studies (Gupta and Varsheney, 2000). These genetic markers exhibit hypervariabilty and are easily detected using PCR and two unique primers (Powell et al., 1996). Microsatellite markers are ideal for identification and genetic fingerprinting of plants because of the high levels of polymorphism they possess (Datta et al., 2010). Based on searches of the literature and GenBank, there is a dearth of microsatellite markers that can be applied to the genus *Viburnum*. In this study, we created a genomic CA-enriched library from *V. dilatatum* ‘Asian Beauty’ using a biotin enrichment protocol (Wang et al., 2007). From the library, eleven polymorphic markers were

developed and used to characterize a group of *V. dilatatum* accessions and cultivars. It is envisioned that these markers will be useful in discerning taxonomic relationships, and will be utilized in future efforts to track and identify the origins of *V. dilatatum* plants within populations that have escaped cultivation.

Materials and Methods

Genomic DNA from unopened flower buds or young leaves of 16 samples (Table 1) was extracted using DNeasy® Plant Mini Kit (Qiagen, Germantown, MD) and quantified using a NanoDrop® ND-1000 ultraviolet-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The quality of DNA was visually assessed on 2% agarose gels, stained with ethidium bromide, and visualized in a 2000 Gel Doc System (Bio-Rad Laboratories, Hercules, CA). Microsatellite sequences were attained from the genomic library and enriched for (GT)n using biotin hybridization based on previous methods by Wang et al. (2007). DNA (2.5 µg) was digested at 37°C for 5 min with AluI, HaeIII, RsaI and StuI to generate blunt-end fragments that ranged from 250 to 800 bp. Fragments were ligated to SNX linkers and recovered utilizing the SNX forward primer adaptors: 5′ CTAAGGCCTTGCTAGCAGAAGC 3’ and 3’AAAAGATTCCGGAACGATCGTCTTCGp 5’ (Hamilton et al., 1999). Column-purified PCR products were hybridized to (GT)12 biotinylated oligonucleotides to enrich fragments that contained (GT)n microsatellite sequences (QIAquick PCR purification Kit, Valencia, CA). Streptavidin Magnesphere® Paramagnetic Particles (Promega, Madison, WI) were used to capture the biotinylated desired motifs (Hamilton et al., 1999; Wang et al., 2004; Wang et al., 2007). The enriched PCR fragment products were column-purified and ligated to the EcoRV digested pBluescript II SK (+) vector, (Stratagene, Hanover, MD) at an 8:1 insert to vector ratio. The vector and ligated enriched SSR-containing fragments were transformed into Escherichia

coli TOP 10 Electrocomp™ cells (Invitrogen, Carlsbad, CA) using the Gene Pulser Xcell® Electroporation System (Bio-Rad, Hercules, CA), and then grown in petri dishes containing Luria-Bertani-ampicillin (LB) medium, and the indicator IPTG and X-gal following the manufacturer's recommendations. The petri dishes were then incubated overnight at 37°C and evaluated for color expression and cell growth (Sambrook et al., 1989).

Detection of putative microsatellites within the cloned DNA fragments was completed as previously described (Wang et al., 2007) with minor modifications. Cells from white colonies with putative inserts were placed in a 10µl PCR reaction mixture containing 1× PCR gold Buffer, 0.2mM dNTPs, 2.0 mM MgCl2, 0.3 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 0.25 mM each of the following three primers: T3, T7, and (GT)12, and sterile water. Conditions during PCR were: 1 cycle at 96°C for 2 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min and 1 cycle of 72°C for 1 min. PCR amplicons were visualized on a 2% agarose gel containing ethidium bromide. Amplicons with a DNA smear were considered putatively positive for having a microsatellite insert (Wang et al., 2007). Colonies of interest were collected and duplicated in 100 □l of LB-ampicillin freezing medium (Sambrook et al., 1989). Plasmid DNA from the putative positive colonies was isolated using a modified alkaline lysis method, and were sequenced (ABE Big-Dye version 3.1 terminators) on a Model ABI 3730XL capillary electrophoresis DNA sequencer (Applied Biosystems).

Putative microsatellite-containing colonies were sequenced (n=198), 47 primer pairs were designed from the resultant sequences using Primer 3.0 (Rozen and Skaletsky, 1998), and synthesized by Integrated DNA Technologies (Coralville, IA). Primer pairs were optimized for PCR using DNA from *V. dilatatum* 'Asian Beauty' and 'Erie'. Microsatellite amplification was

completed using the following 10-□l PCR reaction mixture: 4 ng of genomic DNA, 0.25 μM forward and reverse primers, 2.0 mM MgCl2, 0.2 mM dNTPs, 1×PCR gold Buffer, 0.4 U AmpliTaq Gold DNA polymerase, and sterile water. The following PCR conditions were used: 1 cycle of 94°C for 3 min, and 35 cycles of 94°C for 40 s, 58°C for 40 s, 72°C for 30 s, and 1 cycle of 72°C for 4 min. The PCR products were sized on the QIAxcel Capillary Electrophoresis System (Qiagen) using a 25-500 bp DNA size ladder. Unambiguous reproducible electropherograms were generated from all loci. The raw allele length data was placed into allelic classes using FlexiBinV2, an automated microsatellite binning program that converts raw allele size data into allelic size classes (Amos, et al., 2007). A conservative ± 2bp allele size determination error rate was used due to the 2-bp resolution limitation of the QIAxcel ethidium-based system, to allow reproducibility between various laboratories, and to avoid false inflation of diversity within the data. Of these, the 11 primer pairs (Table 2) that amplified the greatest number of microsatellite alleles were selected for analysis of 16 different accessions of V. dilatatum and related taxa. Allele sizes can vary by only one or two base pairs, which can lead to errors in placing alleles into the proper allelic class when the size is rounded. The resulting allelic classes were used to determine the number of alleles per locus. Twenty-three of the primer pairs were polymorphic and the 11 primer pairs (Table 2) exhibiting the most polymorphisms were selected to analyze 16 different accessions of *V. dilatatum* and related taxa (Table1). After converting the raw data to allelic classes, the number of alleles per locus (A), expected heterozygosity (He), observed heterozygosity (Ho), PIC, and the means of these values for all microsatellite loci were calculated using PowerMarker v3.23 software (Liu, 2005). Deviations from the Hardy-Weinberg equilibrium were calculated using GENEPOP Version 4.0.10 (Rousset, 2008).

Results and Discussion

A total of 81 alleles were observed across the 11 loci. The number of alleles per locus ranged from two (VD006, VD0017) to eleven (VD005, VD012, VD014) and the mean value was 7.36. Observed heterozygosity values were 0 to 0.73 and He ranged from 0.48 to 0.88. All loci, except for VD004, had Ho values that were lower than the He. The majority of our markers did not conform to Hardy-Weinberg equilibrium (Table 2) due to the nonrandom mating and clonal reproduction that occurs with selection of plants for specific characteristics in cultivar development. The PIC values of the microsatellite loci VD006 and VD017 were 0.36, all other loci had PIC values greater than 0.50.

This set of microsatellite markers had a high level of polymorphism and genetic diversity. The ability of a set of molecular markers to delineate between different cultivars depends on the level of polymorphism that can be detected (Datta et al., 2010). In a study of the usefulness of codominant markers in linkage analysis studies, markers were described as being highly informative if PIC values were greater than 0.50, and the majority of this group of markers meet this criteria (Botstein et al., 1980). Additionally, the number of alleles per loci was another important factor in determining the usefulness of markers. Loci with many alleles and PIC values close to 1.0 are most informative and provide the most value in linkage map analyses (Botstein et al., 1980). We intend to use our markers to assess cross transferability to other *Viburnum* species. If future work reveals cross amplification to other *Viburnum* species, these markers are expected to be useful for identifying *Viburnum* species, hybrids and resolving phylogenetic relationships.

Correct identification of plant species and hybrid parentage is essential for breeding programs and patent protection. Moreover, early and accurate identification of invasive species could be a useful tool in combating plant invasiveness. As more ornamental plant cultivars, including

selections that offer reproductive sterility, are developed and become available, germplasm identification will become more complex. Inaccurate identification of plants can be problematic throughout botanic gardens and commercial nurseries in the U.S. (Pryer et al., 2010). Using plastid sequences, Pryer et al. (2010) found that most specimens of a popular fern marketed as Cheilanthes wrightii were in fact the prolific self-regenerating species, *C. distans*. Accurate identification can reduce mislabeling in the nursery, and assist in germplasm management. Moreover, accurate and early identification is important when investigating plant species with invasive potential. Before establishment in the environment, many invasive species undergo lag periods of varying length, during which, time between the species' initial arrival and rapid colonization of a new area may be delayed. A study of alien woody plant species revealed that 51% of all plants had lag periods extending up to 200 years, with shrubs having a mean lag period of 131 years (Kowarik, 1995). Conversely certain invasive plant species, such as *Schefflera actinophylla*, presented a short lag period of only 17 years (Daehler, 2009). Microsatellite markers have become a useful tool for identifying plants in the ornamental horticultural trade, as well as for food crops (Bassil et al., 2010; Wadl et al., 2008).

Accurate characterization and identification of some *Viburnum* species, however, may be confounded in the absence of distinct morphological characteristics presented by fruit and flowers making *V. dilatatum* and its cultivars and hybrids difficult to identify while in their juvenile stage. Indeed, *Viburnum* species generally have juvenility periods lasting from one to three years (Thomas G. Ranney, personal communication). If genetic tools were available, both the environmental lag time and juvenility periods (before seedlings mature and produce seeds) would be ideal times during which potential rogue plants could be detected and screened. Genetic fingerprinting with molecular markers provides an efficient and effective alternative to

plant characterization using biological markers, which are limited through transitive influences of both the environment and developmental stages of the plant (Romero et al., 2009). Indeed microsatellites have been found to have utility in studying invasive plant species (Pashley et al., 2006).

In conclusion, our microsatellite markers have much potential for identifying *V. dilatatum*, its cultivars, and possibly its hybrids. We expect that this set of microsatellite markers will help provide DNA fingerprints for future cultivars, be useful in marker-assisted selection, gene mapping, population genetics, and identification of *Viburnum* species with invasive potential. Furthermore, these loci may provide insight into the work on phylogeny by Winkworth and Donoghue (2005) and help refine the current sections within *Viburnum*.

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Appendix 2: Tables

Table 2-1. *Viburnum dilatatum* with other taxa and their sources.

Species/cultivars/forma analyzed	Specimen origin/accession number[z]
V. dilatatum	NA 56670
V. dilatatum	NA 80690
V. dilatatum 'Asian Beauty'	NCSU
V. dilatatum 'Asian Beauty'	MA 503-99*1 L-31/52-07
(*V. japonicum* × *V. dilatatum* 'Catskill') Chippewa	NCSU
V. dilatatum 'Erie'	NCSU
V. dilatatum 'Erie'	MA 784-2005*1 76-10
V. dilatatum 'Henneke'	MA 153-2004*1 M-43/12-47
V. dilatatum forma *hispidum*	AA 1120-86 *B
V. dilatatum 'Iroquois'	MA 640-2006*1 FF-016/70-95
V. dilatatum forma *pilosulum*	NA 55216

Table 2-1. Continued.

Species/cultivars/forma analyzed	Specimen origin/accession number[z]
V.*dilatatum* forma *pilosulum*	NA 66267
V. dilatatum forma *pilosulum*	AA 77-90 *B
V. dilatatum forma *pilosulum*	AA 36-72 *A
V. dilatatum 'Michael Dodge'	NCSU
(*V. japonicum*× *V. dilatatum*) Fugitive	NCSU

[z]Specimen origin: AA = Arnold Arboretum of Harvard University, Jamaica Plain, MA; MA = Morton Arboretum, Lisle, IL; NA = National Arboretum, Washington, DC; NCSU = Department of Horticultural Science, North Carolina State University, Raleigh, NC.

Table 2-2. Characterization of 11 *Viburnum dilatatum* microsatellite loci.

Locus[z]	Primer sequence (5′-3′)	Repeat motif	T_m (°C)	Allelic size (bp)	*A*	H_e	H_o	PIC
VD003	F: TGGCTCAGATGCATTGAAGAATAG	$(CA)_{12}$	58	105-143	6	0.61	0.19[y]	0.58
	R: GCTGCATGCATCTTCAAATAGG							
VD004	F: GCTGCATGCATCTTCAAATAGG	$(AC)_{16}$	58	108-156	5	0.70	0.73[y]	0.66
	R: ATATCTCGAGGGAGACTGCAACAG							
VD005	F: TTTTAAAACTTTGCACCCTTGCAC	$(CA)_7$	58	115-178	11	0.87	0.72	0.81
	R: AGAATAAAGTCCAGCTCCCTGACC							
VD006	F: ATAACCATATGCGTGTGTATGTTGG	$(GT)_8$	58	137-141	2	0.48	0.00[y]	0.36
	R: GACGTTGCAGGAGCTTCTTATCTC							
VD009	F: GTTTGGGACATGTTCAGTTCTTCC	$(TG)_{12}$	58	116-163	9	0.79	0.67[y]	0.76
	R: AATGTCAGCAAATCAAAATCCAAAC							
VD012	F: TCGACTCTACATTCACTACCCTCC	$(AC)_{16}$	58	128-174	11	0.85	0.47[y]	0.84
	R: CATACGGGTATACGCACACATGC							

Table 2-2. Continued.

Locus[z]	Primer sequence (5′-3′)	Repeat motif	T_m (°C)	Allelic size (bp)	*A*	H_e	H_o	PIC
VD014	F: GCAAACCAAACCACACAAACAC	$(CT)_6$ $(CA)_7$	58	142-204	11	0.88	0.56[y]	0.87
	R: ATCTAGGTCGGCTGCTACTGATTG							
VD016	F: TACCCCTCACAAACACAAACACTG	$(AC)_{12}$	58	71-129	10	0.81	0.60[y]	0.79
	R: AACAATAATGGTGTGGGGTGTTG							
VD017	F: ACCAACCCAATTGCTCAATATCAC	$(AC)_6$	58	165-170	2	0.48	0.00[y]	0.36
	R: GGTTGTCCGCCAGAAGTAGTAGTG							
VD018	F: CTTGCTCGATTTCCCTTATTTGTC	$(CA)_{16}$	58	93-112	4	0.60	0.50[y]	0.54
	R: ATCTCAAGCAAGTCTCACTCCCTC							
VD019	F: AAAGTTGCAAATTACACGCTGATG	$(TG)_{16}$	58	125-167	10	0.86	0.60[y]	0.84
	R: TACCTCCAATTTCACGGTTCTCTC							

Chapter 3. Population genetics of the previously uncharacterized species *Viburnum rufidulum*

Abstract

Viburnum rufidulum is a deciduous shrub with commercial horticultural appeal that is native to North America and has not been genetically characterized. The plant has a unique and attractive red pubescence on leaf buds and petioles, but is also morphologically similar to the non-native and popular ornamental shrub species *V. dilatatum*. These horticultural traits make *V. rufidulum* a good candidate for use within ornamental plant breeding programs and a landscape alternative plant to supplant use of the potentially invasive, non-native species of *V. dilatatum*. In this study, seven microsatellite loci were used to elucidate genetic diversity and population structure of 235 *V. rufidulum* individuals from 17 collection sites located in three counties in Tennessee and one in Kentucky. The observed and expected heterozygosity means were 0.49 and 0.80, respectively, and significant deviation between these values suggested population structure. Population genetic structure and phylogenetic analyses revealed two separate genetic clusters. The F_{st} [fixation index] was 0.087 and indicated a moderate, but significant level of genetic differentiation. Furthermore, most of the genetic variation was found among individual trees rather than among populations, which was also reflected in the low level of gene flow N_m [gene flow] detected. A Mantel test revealed a positive correlation between genetic and geographical distance (R^2 [coefficient of determination]= 0.042, *P* [probability]= 0.010), which suggests that the low level of gene flow and differentiation is a result of habitat fragmentation. The microsatellites developed herein will be useful in breeding programs, valuable in assessments of germplasm, and will help in future studies of *V. rufidulum* populations.

Introduction

Viburnum rufidulum (Rusty Blackhaw) is a member of Adoxaceae, which includes the genera *Sambucus*, *Sinadoxa*, and *Adoxa*. Members of the genus *Viburnum* occur worldwide and

are estimated to include approximately 170 species (Winkworth and Donoghue, 2004). *Viburnum rufidulum*, which is one of 12 species native to North America (USDA, 2008), is a deciduous, woody shrub or small tree with block-like bark that is capable of growing to a height of 3.1 to 9.1 meters (m) (Dirr, 2007). Plants produce small white flowers that are arranged as a cyme and flowering occurs in late spring, attracting a variety of pollinators. *Viburnum rufidulum* is self-incompatible species with a predominantly outcrossing mating system, and the primary pollinators of this genus are syrphid flies, wasps, beetles and native bees (Miliczky and Osgood, 1979). The trees produce attractive berries in late summer, which begin as pale-green fruits, and darken to almost black- purple in late fall. Fruits contain abundant lipids that are nutritionally superior to carbohydrate-rich mast and are an important source of food for both local and migratory birds and small mammals (Meyer and Witmer, 1998). The species can be identified by the red pubescent leaf buds, which are easily seen in late fall and winter and occur on the petioles and abaxial sides of leaves. This abundant pubescence gives *V. rufidulum* its common name of Rusty Blackhaw and the attractive and unique rust colored pubescence distinguishes it from all other native *Viburnum* spp. (Dirr, 1998). For example, *V. prunifolium* is very similar to *V. rufidulum*, but is easily identified because it lacks reddish pubescence on leaves. *Viburnum rufidulum* is often found growing as an understory tree on limestone outcroppings in shallow, xeric environments (Homoya, 1987). The native range of *V. rufidulum* extends from the eastern southern United States westward, traversing the Gulf States (spreading north into the southern portions of Illinois, Indiana, Missouri, and Ohio), and extending to eastern sections of Kansas, Oklahoma, and Texas (http://plants.usda.gov/core/profile?symbol=VIRU).

Viburnum species are of great interest to the ornamental horticultural community and are an economically important genus (Clement and Donoghue, 2012; Dean et al., 2011). Many non-

native *Viburnum* spp. and cultivars are popular and ubiquitous in landscapes and gardens throughout North America. However, the introduction of non-native species is a major threat (second only to habitat loss) to the biodiversity of native plants in the environment with many invasions created deliberately through the anthropogenic introduction of exotic plant species (Vitousek and Walker, 1989; Gurevitch and Padilla, 2004). Additionally, urbanization has led to fragmentation and loss of habitat. Fragmentation can result in spatial isolation, which promotes inbreeding and the reduction of gene flow into naturally occurring populations (Den and Oostermeijer, 1997). Moreover, the genetic differentiation, gene flow, and levels of heterozygosity of native plant populations are of interest to biologists and serve as indicators of local biodiversity and species richness of plant and animal communities (Lindenmayer et al., 1999; Kati et al., 2004). *Viburnum rufidulum* has not been genetically characterized nor, does information concerning population structure and diversity exist. Individual trees located within a sampling area may display little or no difference in morphology, therefore molecular studies can provide valuable insight to the genetic composition of populations. Knowledge of the genetic diversity and variation of plant species can assist in determining long-range survival, and is often the first step taken to manage and conserve native populations. Moreover, genetic characterization of native North American species can become important to successful conservation efforts if required (Holsinger et al., 1991; Hamrick and Godt, 1996; Newton et al., 1999).

Microsatellite loci also referred to as simple sequence repeats (SSRs), are a class of co-dominant markers that are applicable to genetic diversity and population studies. These markers are tandemly repeating nucleotide segments of DNA that are situated throughout the genomes of most eukaryotic species (Powell et al., 1996). The number of repeats occurring at these loci is

extremely variable due to the high rate of mutation that can take place when polymerase slippage occurs during DNA replication (Bruford and Wayne, 1993; Peakall et al., 1998). The hyper-variability of repeat length at the microsatellite loci can be amplified easily using polymerase chain reaction (PCR) and two unique primers (forward and reverse) designed from the flanking regions of the repeats. Microsatellite loci have proven to be valuable genetic markers in studies involving plants because of the high level of polymorphism that results from this hyper-variability (Gupta et al., 1996). Furthermore, microsatellite loci are capable of discerning polymorphisms even in species that have low levels of genetic diversity (Peakall et al., 1998). Thus, microsatellite loci reveal high allelic diversity and afford a means of accurately characterizing individuals in natural populations (Chase et al., 1996). These markers are often used for population genetic studies that involve closely related individuals and are a valuable tool for studying gene flow, inbreeding and levels of differentiation between populations (Arnold et al., 2002; Zhang and Hewitt, 2003; Hadziabdic et al., 2012; Moura et al., 2013).

Here, we genetically characterized the native North American species *V. rufidulum*. To our knowledge, this is the first study to examine the genetic diversity and population structure of *V. rufidulum* in Tennessee and Kentucky. The specific objectives of this study were to utilize microsatellite markers to (1) quantify genetic diversity; (2) analyze population structure of four naturally occurring populations of *V. rufidulum;* and (3) characterize patterns of gene flow.

Methods and Materials

During late winter and early spring of 2011 through 2013, a total of 235 samples of *V. rufidulum* leaf and flower buds were randomly collected from plants growing at 17 sampling areas located in Tennessee and Kentucky (Fig. 1). There were six collection sites in Knox and Loudon counties Tenn. and two sites in Anderson County, Tenn. Samples were collected from

three sites in Fayette County, Ky., for a total of 17 subpopulations (Table 1). Identification of samples was based on morphological characteristics of the pubescent leaf buds (unique to *V. rufidulum*) and rectangular or block-like bark plates on the trunk. Leaf buds were collected from individual trees that were spaced at least 1.2 m apart and trees were temporarily tagged to prevent resampling of individuals. A total of 235 individual trees were selected and leaf and flower buds collected from each tree. The sample sizes from sites ranged from 2 to 33 individual trees with each sample being represented once in this study. The VA, FR2, and PNS sites yielded only two to four trees each. We elected to include these small populations in the study, as each of those collection sites is undergoing significant disturbances from urbanization or invasive species. In addition, small population sizes did not affect estimates of gene diversity in former studies (Nybom and Bartish, 2000; Semaan and Dodd, 2008). Furthermore, when the smallest populations (VA, PNS, and FR2) were grouped into a population that included all Knox County populations, the levels of diversity and differentiation did not differ (data not shown). Individual samples were labeled according to the tree from which they were collected, and global positioning system (GPS) coordinates were recorded (Table 1). Plant materials were transported from the field to laboratory on ice and stored at -80°C until genomic DNA was extracted.

DNA extractions and development of SSR markers

Genomic DNA was extracted from unexpanded leaves or closed flower buds of specimens with a Qiagen DNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA). DNA was evaluated for purity and quantified with a NanoDrop® ND-1000 Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Additionally, each sample was assessed for the presence of a strong band of genomic DNA (to ensure quality) using a 2% agarose gel

stained with ethidium bromide. The gel image was recorded with a 2000 Gel Doc System (Bio-Rad Laboratories, Hercules, CA, USA).

A small insert genomic DNA library was constructed and enriched for $(CA)_n$ simple sequence repeats (SSR) with a method developed by Wang et al. (2007). Briefly, 2.5 µg of genomic DNA of *V. rufidulum* (accession #24 collected in Anderson County, TN) was digested using the restriction enzymes *AluI, HaeIII, RsaI*, and *StuI* (0.5 U/ µl of each enzyme). The digestion was completed at 21°C for 45 min, which produced blunt-end fragments that ranged in size from 250 to 800 base pairs (bp) when separated on 2% agarose gel and visualized on a 2000 Gel Doc System. Enzymes were inactivated at 70°C for 20 min. The digested fragments were ligated to the SNX linkers: 5' CTAAGGCCTTGCTAGCAGAAGC 3' and 3' AAAAGATTCCGGAACGATCGTCTTCGp 5' using PCR conditions described by Hamilton et al. (1999). The ligated PCR products were column-purified (QIAquick PCR purification Kit, Valencia, CA, USA) and hybridized to $(GT)_{12}$ biotinylated oligonucleotides. This procedure allowed the recovery of enriched $(CA)_n$ –containing PCR products using Streptavidin MagneSphere® Paramagnetic Particles (Promega, Madison, WI, USA) (Hamilton et al., 1999; Wang et al., 2007). Following column purification, the enriched PCR fragment products were ligated to the *Eco*RV-cut pBluescript SK II (+) vector, (Stratagene, Hanover, MD, USA) using an 8:1 DNA fragment to vector ratio. The vector and ligated enriched SSR-containing fragments were transformed into *Escherichia coli* TOP 10 Electrocomp™ cells (Invitrogen, Carlsbad, CA) using the Gene PulserXcell® Electroporation System (Bio-Rad, Hercules, CA, USA). The transformed bacteria were plated onto Luria-Bertani-ampicillin (LB) agar plates with 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-gal) and β-D-galactoside (IPTG), and incubated at 37°C overnight following a procedure by Sambrook et al. (1989). The pBluescript SK II (+) vector

contains a multiple cloning site (MCS) and the *lacZ* gene that normally expresses β-galactosidase, which converts X-gal from white to blue. However, if the bacterial colonies are successfully transformed with the plant DNA fragments, and inserted into the MCS, the *lacZ* gene is interrupted and prevented from expressing β-galactosidase, and recombinant bacteria and subsequent colonies remain white. White colonies were selected, and grown in liquid Luria-Bertani (LB) medium overnight at 37°C. One µl of cell suspensions of *E. coli* from selected colonies containing *Viburnum* genomic DNA were added to a 10 µl PCR reaction containing the following: 1× PCR gold Buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.4 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 0.25 mM each of the following three primers T3, T7, and $(GT)_{12}$, and sterile water (Wang et al., 2007). The thermal cycle conditions were as follows: one cycle at 96°C for 2 min, followed by 35 cycles of 94°C for 3 min, 55°C for 40 s, 72°C for 30 s and one cycle of 72°C for 4 min. Amplified PCR products were visualized on a 2% agarose gel and those primers that supported amplification and produced a smear in the gel (indicative of multiple products) were identified as those that may contain a microsatellite insert of the desired CT motif (Wang et al., 2007). Colonies with putative microsatellites were selected and replicated in 100 µl of LB-ampicillin freezing medium, and grown at 37°C (Sambrook et al., 1989). Plasmid DNA from the duplicated colonies was sequenced using T3 and T7 primers, on an ABI 3730XL capillary electrophoresis DNA sequencer (Applied Biosystems, Foster City, CA, USA). Following sequencing, repeats were located with Imperfect SSR Finder (Stieneke and Eujayl, 2007), and vector flanking regions were trimmed from the insert using VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/).

Forty-seven forward and reverse primer pairs were designed using Primer 3.0 (Rozen and Skaletsky, 1998) with the following optimum parameters: oligonucleotide length = 20 bp,

primer GC% content of 50% and an annealing temperature of 56ºC. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer pairs were optimized for PCR using genomic DNA from 20 *V. rufidulum* samples and a 10-µl PCR amplification reaction consisted of the following: 4 ng of genomic DNA, 2.5 µM forward and reverse primers, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1× PCR gold Buffer, and 0.4 U AmpliTaq Gold DNA polymerase. The thermal cycler conditions were as follows: 1 cycle of 94°C for 3 min, and 35 cycles of 94°C for 40 s, 58°C for 40 s, 72°C for 30 s, and 1 cycle of 72°C for 4 min. The amplicons were sized on the QIAxcel, Capillary Electrophoresis System (Qiagen, Valencia, CA, USA). Of the 47 primer pairs, seven microsatellite markers were optimized and developed (Table 2). The seven primer pairs were used to amplify DNA from all 235 *V. rufidulum* samples included in the study. Reactions that failed to produce amplified products were repeated twice before an individual was considered null at that locus.

Data Analyses

Prior to analyses, all raw allele length data bp sizes were placed into allelic size classes using the automated binning program FlexiBinV2 (Amos et al., 2007). The QIAxcel Capillary Electrophoresis System (Qiagen) was used for analyses of PCR products and a conservative ± 2-bp allele size determination error rate was utilized to permit reproducibility of this data. However, as found in previously published studies (Wadl et al., 2011; Hadziabdic et al., 2012) binned data results were similar to unbinned data results.

To examine the genetic diversity of *V. rufidulum* in the sample areas, 235 individual trees were selected and designated according to which county they were collected: Anderson, Knox, Loudon (Tenn.) and Fayette (Ky.). We measured the expected heterozygosity (H_e), observed heterozygosity (H_o), total heterozygosity (H_T), and the number of alleles (A) for each

microsatellite locus across all populations using FSTAT Version 2.9.2.3 (Goudet, 1995; 2001). Additionally, allelic richness was calculated using a minimum sample size of two individuals (four genes), which was found at the VA site, utilizing a hierarchical rarefaction method in HP Rare 1.0 software (Kalinowski, 2005). Polymorphic information content (PIC) was estimated with the program CERVUS Version 3.0 (Kalinowski et al., 2007). And GenAlEx Version 6.4.1 (Peakall and Smouse, 2006) was used to estimate gene flow (N_m).

Genetic differentiation or fixation index (F_{st}), inbreeding (F_{is}), and overall inbreeding coefficients (F_{it}) are collectively known as *F* statistics (Wright, 1931; 1951) and have been widely used in population genetics to measure genetic differentiation and heterozygote deficiency or excess within populations and among groups of populations. These parameters were calculated with a 95% confidence level using the program FSTAT Version 2.9.3 (Goudet, 2003). GenAlEx Version 6.41 (Peakall and Smouse 2006) was utilized to measure gene flow (N_m) over all populations for each locus. All loci were tested by randomization for the levels of significance ($P < 0.05$) for deviation from Hardy-Weinberg equilibrium (HWE) within each designated population. HWE was calculated with a modified Markov-chain method described by Guo and Thomson (1992) with Fstat using 1,000 permutations.

Genetic structure and differentiation between *V. rufidulum* populations were measured using Structure version 2.3.4 (Pitchardet al., 2000) a Bayesian model approach. Structure assigns individuals to different genetic clusters based on the allele frequencies occurring at various loci, thus clustering in a way that Hardy-Weinberg equilibrium is achieved (Pitchardet al., 2000). *K* is a measure of the number of statistically different populations within the data set. Ten iterations/independent runs for each value of *K* (*K* = 1 to 8) were employed. A burn-in period of 100,000 was utilized as well as 100,000 Markov Chain Monte Carlo replications after burn-in, as

these are long enough to achieve consistent results (Pritchard et al., 2000). The admixture (mixed ancestry among individuals that may be present) and the correlated allele frequencies (allele frequencies may be similar among the populations) models were also employed as they can detect admixture that has occurred in the past, and are capable of detecting more subtle population subdivisions (Falush et al., 2003). The most probable value of *K* was selected based on the calculation of [Pr(X\K)] (probability distribution), and by estimating the number of populations with the *ad hoc* statistic Δk (Evanno et al., 2005) through Structure harvester Version 0.6.1 (Earl and von Holdt, 2012). The hierarchical distribution of genetic variation was partitioned into different levels and was characterized by analysis of molecular variance (AMOVA) using Arlequin Version 3.5.1.2 (Excoffier and Lischer 2010).

The following two AMOVA were performed: the first analysis placed all individuals into one hierarchical group, and the second partitioned *V. rufidulum* individuals into 17 subpopulations based on their collection sites. Genetic structure is inferred by evaluating allelic frequencies and the significance of the different levels of all hierarchical genetic structures is verified using non-parametric permutation methods (Excoffier et al., 1992) in which 99,999 permutations were incorporated.

To validate genetic differentiation of *V. rufidulum* populations, 10,000 permutations were employed to calculate Nei's (1972) pairwise genetic distances (computed as F_{st} according to Slatkin (1995)) for all pairs of populations using program Arlequin ($P \leq 0.05$). The summary statistic F_{st} was used to quantify gene differentiation and the hierarchical partitions between, among, and within groups or populations (Excoffier, 2007; Holsinger and Weir, 2009; Kronholm et al., 2010). A Mantel test (Mantel, 1967) for isolation by distance using GenAlEx Version 6.5 (Peakall and Smouse, 2006) was used to examine the correlation between genetic and geographic

distance of all loci in all populations and was performed incorporating 9999 permutations. The results of the Mantel test supports the findings of genetic structure. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram was created using standard genetic distance (Nei, 1972), bootstrap analysis was generated with the program Populations 1.2.32 (Langella, 2002), and used to visualize the relationships of all individuals in each population. Support of the branches was approximated with 500 bootstrap randomizations. A consensus tree with support was then generated and viewed using Treeview 1.6.6 software (Page, 1996).

Results

In this study, we evaluated the genetic diversity of *V. rufidulum* by genotyping 235 individuals from four separate and naturally occurring populations using seven polymorphic microsatellite markers. The means across all loci were tested for the number of alleles (A), expected heterozygosity (H_e), observed heterozygosity (H_o), total heterozygosity (H_T), gene flow (N_m), and *F* statistics (Table 3). The seven microsatellite markers amplified about 20 alleles per locus. Locus VR008 revealed the fewest (8) and VR004 revealed the highest number of alleles (35). With the exception of VR004 ($H_e = 0.83$, $H_o = 0.90$), VR008 ($H_e = 0.78$, $H_o = 0.97$), and VR012 ($H_e = 0.62$, $H_o = 0.60$), HWE was rejected for the remaining loci due to heterozygote deficiency ($P < 0.05$). The allelic richness over all loci per collection site (corrected by rarefaction) ranged from 2.63 to 3.50 and had a mean of 3.40. The greatest degree of gene diversity and polymorphism was found at locus VR004 ($H_e = 0.83$, PIC = 0.90) and the least was detected at locus VR011 ($H_e = 0.67$, PIC = 0.70)

Genetic structure was also analyzed via *F* statistics and the mean inbreeding coefficient as measured by F_{is} was 0.21, which supported the finding of an overall deficiency of heterozygotes. The deviation from HWE for this group of subpopulations, as measured by F_{is},

indicated that all values were positive for all loci except VR004 (-0.24), VR008 (-0.53), and VR012 (-0.06), reflecting an excess of heterozygosity at those three loci. HWEs were significant for an excess of homozygosity (P<0.05) at loci VR005, VR011, VR028, and VR043, therefore, equilibrium was rejected. Additionally, the total inbreeding coefficient (F_{it}) was high (0.47) and indicated an excess of homozygotes. The values for loci VR004, VR008, and VR012 were negative due to the aforementioned excess of heterozygosity. The F_{st} values across all loci ranged from 0.16 to 0.30, with a mean value of 0.21. The least amount of differentiation was found at the VR008 locus and the most was observed at loci VR028. Gene flow estimations over all populations for each locus ranged from 0.58 to 1.77, with a mean of 1.05 (Table 3).

STRUCTURE analysis indicated that the highest value of ΔK occurred at K = 2 (Fig. 2). Two genetic clusters emerged with subpopulations PNS, FR2, TL1, TL2, TL4, and OL being admixed almost evenly, whereas the remaining subpopulations were more uniform in genetic composition. To analyze the hierarchical partitioning of genetic variation, an AMOVA was implemented using the program Arlequin (version 3.5.1.2) (Table 4). The AMOVA analysis was significant (P<0.05) and revealed that the greatest amount of genetic variation was found among individuals (91%), rather than between populations (9%). The F_{st} was 0.087 with P value < 0.05, indicating genetic structure among the populations. Molecular variance was significant among groups, among populations within groups, among individuals within populations and within individuals (F_{is} = 0.39, F_{sc} = 0.10, F_{ct} = 0.03, and F_{it} = 0.47) (Table 4). The F_{is} and F_{it} indicated a deficit of heterozygotes due to non-random breeding at the population level.

Considering all populations, most of the pairwise F_{st} values were significant and 86% of the values were > 0.10 (Table 5). The greatest differentiation occurred between populations FR1 and TL5 (0.35), and FR1 and VA (0.45). The least amount of variation (0.02) was found between

RR1 and OL. The UPGMA analysis (Fig. 3) clustered the 17 subpopulations into two major clades, which corroborated the structure analysis. Bootstrap support was low, the only nodes that demonstrated values greater than 50% were WC1 and WC2 and also WEB and RR1. Furthermore, the Mantel test (Fig. 4) revealed a significant correlation between genetic and geographical distance ($R^2 = 0.042$, $P = 0.010$).

Discussion

The high level of genetic diversity found at the 17 collection sites of *V. rufidulum* are similar to other studies involving the population genetics of outcrossing trees (Hamrick et al., 1992; Hamrick and Godt, 1996; Furches et al., 2013). The average genetic diversity of outcrossing plant species in other studies employing microsatellite markers was 0.65 (Nybom, 2004). However, our study revealed an average gene diversity value, which was 0.79, and was higher than reported for other studies of woody species (Hornero et al., 2001; Kikuchi and Isagi, 2002). High levels of diversity can be expected in plants that have outcrossing mating systems, are long-lived, and have more than one generation that coincides simultaneously with other generations at the same location (Hamrick and Godt, 1996; Till-Bottraud and Gaudeul, 2002; Nybom, 2004; Marra et al., 2013). In a study of the endangered woody species *Liriodendron chinense*, maintenance of genetic variation was attributed to high levels of outcrossing (Feng et al., 2010; Li et al., 2014). The high mean value for gene diversity (0.74) within these populations could be explained by the extensive geographic range of *V. rufidulum*. Additionally, gene diversity contributes to the stability of populations when adverse conditions occur, such as disease outbreaks, and to successful adaptation to and survival during environmental changes (Leonard, 1969; Freeman and Herron, 1998).

The highest levels of diversity in our study were at sites that boasted large areas of unspoiled habitat, which included sites in Anderson and Loudon counties in Tenn., and Fayette County Ky. whereas the least gene diversity was found at the FR1 an FR2 sites in Knox County, Tenn. Interestingly, while the vast area at the Forks of the Rivers sites seemed like ideal habitat for *V. rufidulum*, few trees were growing there and those that were discovered were growing in tight clusters. There was extensive growth of *Ligustrum sinense* (Chinese privet), an invasive Asian species, observed in close proximity to the *V. rufidulum* trees. The presence of Chinese privet seemed to anecdotally be related to the loss of diversity at this site. Privet invasion of natural habitats is detrimental to native species and results in the crowding of native understory plants, decreased indigenous plant abundance, and reduction in seedling recruitment (Wilcox and Beck, 2007; Hanula et al., 2009). Invasion of non-native species constitute a major threat to biodiversity, being second only to environmental fragmentation and habitat loss (Coblentz, 1990; Shigesada and Kawasaki, 1997). When fragmentation is coupled with inbreeding, heterozygosity levels decline (Young et al., 1996).

An overall heterozygote deficit could indicate that individuals within populations of *V. rufidulum* are not panmictic. As with other obligate outcrossing species, the overall deficit of heterozygotes and deviation from HWE may be explained by the ability of this species to mate with half-sibs. This lack of nonrandom mating can result inbreeding among closely related individuals. Similar findings have been observed for flowering dogwood (*Cornus florida*) (Hadziabdic et al., 2012), and in the herbaceous alpine plant *Campanula thyrsoides* (Ægisdóttir et al., 2009).

There is evidence that fragmentation has a profound effect on patterns of pollination (Rathcke and Jules, 1993; Aizen Feinsinger, 1994; Didham et al., 1996). Young et al. (1996)

found that these alterations can change mating system parameters and have an effect on levels of heterozygosity. Levels of heterozygosity respond more rapidly than gene diversity (Young et al., 1996), which can explain the high level of gene diversity found in this study.

STRUCTURE analysis partitioned *V. rufidulum* from the 17 collection sites into two principal genetic clusters; however, admixture of the two genetic types was noted within almost all of the collection areas. There was a lack of bootstrap support, but this could be related to the fact that a few loci were used. The presence of admixture within the two genetic clusters, as inferred by the program STRUCTURE, implied that interbreeding or sharing of alleles has occurred between the two clusters at some time. WC1 and WC2 displayed a strong cluster, but admixture of the two populations could be observed. These two collection sites were in close proximity to each other and were probably one population at some time according to the small amount of differentiation revealed in the pairwise F_{st} value. In addition, TL5 had strong support for clustering with no genetic variation between the individuals according to the STRUCTURE analysis. The TL5 population was isolated from the other five TL collection sites by approximately 0.80 km, which may account for the strongly supported genetic cluster. All of the other populations displayed an equal distribution of alleles. Interestingly, the two trees found at the VA site were mature and growing in a small patch of woods within a park. It seemed likely that seedlings and other trees might be found, but there were no others. However the two trees shared a similar genetic clustering, (it is not known if they are clones). Therefore mating might be likely to be unsuccessful considering the predominantly outcrossing mating system of the species, as fertility barriers can exist to prevent inbreeding in closely related plants. Small population size is one of the deleterious effects of habitat fragmentation (Lienert, 2004).

Some woody plants are capable of maintaining high levels of genetic diversity because of their large size and capacity to produce copious amounts of pollen and seeds that can be dispersed over long distances, thereby driving gene flow (Hamrick, 2004; Vranckx et al., 2011). On the other hand, low levels of gene flow in some woody species have been attributed to small population sizes and habitat fragmentation (Young et al., 1996; Semaan and Dodd, 2008; Li et al., 2114). Gene flow values in this study were markedly low and appeared to potentially be related to urbanization and destruction of habitat.

Fragmentation of habitat can affect patterns of pollen distribution. For example, while bees are capable of traveling distances of 1.5 to greater than 4.5 kilometers (km) (Petit and Hampe, 2006), bees and other pollinators prefer to travel no more than 100 to 1000 m (Rathcke and Jules, 1993; Lienert, 2004). Additionally, pollinators tend to select large patches of vegetation that offer different flower varieties (Olesen and Jain, 1994). As fragmentation reduces the population size of plants, pollinators are less likely to visit those populations. Therefore, gene flow among existing plant populations is decreased. Long time spatial isolation has been implicated in the reduction of pollen movement that leads to population divergence (Li et al., 2014). This may play a role in the overall moderate F_{st} values and high pairwise F_{st} values between populations of *V. rufidulum*.

The PNS and CP populations are separated by a distance of approximately 2.0 km. However, the area is undergoing continual development of housing, large shopping centers, and the recent addition of an entrance ramp to an interstate. The small stand of trees located at the PNS site were older trees; there were no younger trees or seedlings in that area. Additionally, bulldozers were clearing trees in close proximity to the four remaining trees. The CP subpopulation was more plentiful, and the patch of woods in which trees grew was a designated

trail with no urbanization disturbances. There, the trees were younger and seedlings were plentiful. The destruction of habitat may have had a disruptive effect on a formerly contiguous patch of woods that may have interrupted both pollen flow and seed dispersal. Moreover, these two sites have become genetically differentiated as evidenced by the high pairwise F_{st} value in spite of existing in close proximity to each other. Conversely, the WC1, WC2, RR1, and OL sites were modestly differentiated; these sites were in close proximity, but boasted large, healthy populations of trees. The areas were pristine with no evidence of invasive species. In contrast, both of the FR sites were included in the two highest pairwise differentiation values, which may be a result of competition with an invasive species (Chinese privet) coupled with small population size at those sites. As populations become more isolated, they become more differentiated. High levels of genetic differentiation may occur when population sizes become small and gene flow has decreased as a result of habitat fragmentation (Young et al., 1996).

The overall low levels of gene flow across all loci coupled with high pairwise F_{st} values could reflect fragmentation of a once larger natural range of *V. rufidulum*, which effectively serves as a barrier to pollen flow and seed dispersal. In a study of the endangered Azorean shrub, *V. treleasie*, in which populations were separated by a series of islands in an archipelago, gene flow values were low (0.35-1.02) and F_{st} values were high (0.3-0.5), reflecting the influence of geographical barriers (Moura et al., 2013) on the population structure. The gene flow and F_{st} values in the *V. treleasie* study are similar to those found in our survey of *V. rufidulum*. Spatial isolation serves as great a barrier to gene flow as geographical barriers such as water or mountain ranges.

There is great variation between sites in this study. Therefore, the introduction of unique alleles into existing populations must be occurring. As indicated by the AMOVA analysis,

genetic diversity is being maintained by individuals as opposed to between populations. Gene flow into our sampled *V. rufidulum* populations may be sustained by long-range seed dispersal of birds. *Viburnum* fruit is consumed by myriad bird species (Beal, 1915; Martin et al., 1951; Rybczynski and Riker, 1981). The fruit pulp is lipid-rich and serves as a "nutritionally superior" food source to frugivorous bird species (Meyer and Wetmore, 1998). The distance at which seed dispersal occurs is related to the mode in which different bird species feed, carry, and disperse the seeds (Murray et al., 1994). Most *Viburnum* species produce hundreds of drupes per season (Rybczynski and Riker, 1981), including *V. rufidulum*. Therefore, it can be expected that seeds of an individual would be dispersed close to an established stand when birds gather in large flocks and feed within dense populations of trees. The potential to scatter seeds of related trees in close proximity to each other might potentiate the inbreeding of related individuals. This phenomenon appears to be supported by the high level of inbreeding (F_{is} = 0.21) that is occurring in these populations. In addition, habitat fragmentation is a contributing factor to non-random breeding within populations (Knapp et al., 2001).

Conversely, if migratory birds scatter seeds at greater distances, the introduction of novel alleles into existing populations would be expected to occur. Overlapping of generations also increases diversity within the populations as trees of different ages flower. Because of the prolonged juvenile phase of many trees (delayed reproduction), genetic variation can only be increased by recruitment of new individuals. Novel alleles are therefore introduced to the population on an individual basis and established by the time the trees reach sexual maturity. *Viburnum* species typically have juvenility periods lasting up to three years and therefore do not flower at this time (Thomas G. Ranney, personal communication). This could explain the high level of diversity within the populations included in this study.

Our high pairwise F_{st} indicate that the collection sites are differentiated. It's been suggested that diversification resulted from movement of individuals into new geographical areas and once there adaptive radiation occurred (Donoghue, 2006). Furthermore, Donoghue hypothesized that the dispersal of seeds by birds across land barriers lead to reproductively isolated populations in similar geographic areas (2006). Therefore, as habitat becomes more fragmented, might individual collection sites evolve into distinct populations that eventually become reproductively isolated?

In this study we have characterized information on the range of genetic diversity present within native localities previously uncharacterized species, *V. rufidulum.* The level of genetic differentiation and isolation by distance support the low levels of gene flow that is occurring between the various collection sites. The structure of these populations of *V. rufidulum* is likely the result of habitat fragmentation and geographical isolation. Similarity of the genetic clusters suggests that the 17 separate populations are fragmented remnant populations that were once part of a large contiguous population of trees. The microsatellite loci used in this study revealed a large number of informative alleles, making this set of markers useful for evaluating levels of diversity within other *V. rufidulum* populations and potentially other *Viburnum* sp. Future studies would benefit from using additional markers to evaluate more populations across a broader swath of the natural range of *V. rufidulum* and also incorporating collection locales that are separated by geographical barriers.. *Viburnum rufidulum* is important as a native tree that also has attractive ornamental qualities, and we expect this set of markers to be of value to breeding programs that aim to utilize the attractive traits of the tree, such as the rust-colored pubescence, or to promote the use of native plants as an alternative to potentially invasive non-native species.

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Appendix 3: Tables

Table 3-1. Collection site information, identification, sample size and GPS coordinates for 235 *Viburnum rufidulum* trees located in Tennessee and Kentucky.

Collection site	Population Code	Number of trees sampled	GPS coordinates W	N
Anderson County, TN	WC1-Worthington Cemetery	33	36.0289	-84.1222
Anderson County, TN	WC2-Worthington Cemetery	24	36.0288	-84.1242
Knox County, TN	WEB-Webb School	29	35.9232	-84.1170
Knox County, TN	PNS-Pellissippi and Northshore	4	35.8500	-84.0700
Knox County, TN	CP-Cowan Park	14	35.8515	-84.0932
Knox County, TN	FR1-Forks of the River	7	35.9591	-83.8211
Knox County, TN	FR2-Forks of the River	3	35.9593	-83.8209
Knox County, TN	VA-Victor Ashe Park	2	35.9826	-83.9956
Loudon County, TN	TL1-Tellico Lake	12	35.7682	-84.2579
Loudon County, TN	TL2-Tellico Lake	6	35.7650	-84.2647
Loudon County, TN	TL3-Tellico Lake	6	35.7670	-84.2583
Loudon County, TN	TL4-Tellico Lake	15	35.7687	-84.2607
Loudon County, TN	TL5-Tellico Lake	20	35.7668	-84.2552
Loudon County, TN	TL6-Tellico Lake	6	35.7670	-84.2593
Fayette County, KY	RR1-Raven Run	24	37.8843	-84.4030
Fayette County, KY	RR2-Raven Run	15	37.8842	-84.4032
Fayette County, KY	OL-Overlook	15	37.8840	-84.4029

Table 3-2. Characteristics of *Viburnum rufidulum* microsatellite loci that were used to analyze 235 sampled tree specimens collected in Kentucky and Tennessee.

Locus	GenBank Accession Number	Primer sequence (5'-3')	Repeat motif	Expected size (bp)
VR004	KC236414	F:CTACCGCACATATGCACCTAC R:TACAGATCGGGAAGGTGTAAGG	TG_6…TG_7…TGTGT G_2	210
VR005	KC236415	F:TGCTTCCATCTCTTTTCTCTCC R:GTGTGTGGCTGTGTTTGTACG	TG_6…TG_7…GT_7	113
VR008	KC236416	F:CCAAGCCCCATTTTATAAATACC R:CATTCTGCCATTTTATTGAGTCC	$AAGAATTT_2$… $TTAAGAA_2$	128
VR011	KC236417	F:GCATATGTGCACACACGAGAG R:TATGGATTGGGAAGGTGTTAGG	CA_{12}	174
VR012	KC236418	F:CAAAGTGGGCAGAGAAGTAGC R:CATTGTAGAGCACCACAAATTCC	TA_7	245
VR028	KC236419	F:GCTCGTAGCAGGGGTGTGTAT R:CACGCACACTGCACACACT	CA_5…AC_8	116
VR043	KC236420	F:TATGTGAGGATGAAGGTGATGG R:TTACATTCTGGCAGTAGCAACC	CT_7…TC_5…TC_5	213

Table 3-3. Nei's estimation of heterozygosity, and *F* statistics across all *Viburnum rufidulum* loci and averaged over all populations as calculated by FSTAT version 2.9.3. Polymorphic information content was estimated using CERVUS 3.0. Gene flow was estimated with GenAlEx version 6.4.1.

Loci	N_a*	H_o	H_e	H_T	F_{is}	F_{it}	F_{st}	N_m	PIC
VR004	35	0.90	0.83	0.89	-0.24	-0.02	0.18	1.18	0.90
VR005	22	0.12	0.80	0.85	0.82	0.85	0.18	1.21	0.88
VR008	8	0.97	0.78	0.76	-0.53	-0.29	0.16	1.32	0.75
VR011	19	0.34	0.67	0.73	0.35	0.53	0.27	0.67	0.70
VR012	14	0.60	0.62	0.72	-0.06	-0.16	0.21	0.95	0.76
VR028	14	0.32	0.69	0.69	0.34	0.54	0.30	0.58	0.66
VR043	20	0.17	0.80	0.88	0.76	0.80	0.18	1.17	0.88
Mean	18.8	0.49	0.74	0.79	0.21	0.47	0.21	1.00	0.79

* Number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity under Hardy-Weinberg equilibrium (H_e), total expected heterozygosity (H_T). *F* statistics: inbreeding coefficient (F_{is}), overall breeding coefficient (F_{it}), genetic differentiation coefficient (*Fst*), gene flow (N_m), and polymorphic information content (PIC).

Table 3-4. Analysis of molecular variance (AMOVA) for *Viburnum rufidulum* populations occurring in four counties in Tennessee and Kentucky based on microsatellites using ARLEQUIN (version 3.5.1.2).

	d.f.	Sum of squares	Variance component	% of variation	*P* value
Variance partition[a]					
Among populations	8	114.825	0.24865 Va	8.76	<0.0001
Within populations	457	1182.887	2.58837 Vb	91.24	<0.0001
Total	465	1297.712	2.83702		
Fixation index	F_{ST} = 0.087				
Variance partition[b]					
Among groups	3	86.751	0.10554 Va	3.31	<0.0082
Among populations within groups	13	146.849	0.31383 Vb	9.84	<0.0001
Among individuals within populations	218	837.434	1.07072 Vc	33.56	<0.0001
Among individuals	235	399.500	1.70000 Vd	53.29	<0.0001
Total	469	1470.534	3.19009		
Fixation indices	F_{IS}[d] = 0.386	F_{SC}[e] = 0.101	F_{CT}[f] = 0.033	F_{IT}[g] = 0.467	

[a] Analysis with all samples in one hierarchical group.
[b] Analysis of the four counties in which the samples were collected including among groups, among populations within groups, among individuals within populations, and among individuals.
[c] F_{ST} variance among subpopulations compared to the overall variance.
[d] F_{IS} inbreeding coefficient of individuals compared to the population.
[e] F_{SC} variance component found among subpopulations within groups.
[f] F_{CT} variance component found among groups compared to the total variance.
[g] F_{IT} variance of the total population.

Table 3-5. Significance of F_{st} pairwise comparison values of 17 subpopulations of *Viburnum rufidulum* occurring in Tennessee and Kentucky as calculated by the program ARLEQUIN (version 3.5.1.2) above the diagonal and pairwise F_{st} values below the diagonal.

Populations 1-17

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2	0.048		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
3	0.109	0.130		*	*	*	*	*	*	*	*	*	*	*	*	*	*
4	0.153	0.121	0.137		*	*	*	NS	*	NS	*	*	*	*	*	*	*
5	0.111	0.109	0.070	0.187		*	*	*	*	*	*	*	*	*	*	*	*
6	0.153	0.206	0.186	0.313	0.178		*	*	*	*	*	*	*	*	*	*	*
7	0.133	0.208	0.158	0.225	0.188	0.277		NS	*	NS	*	*	*	*	NS	*	NS
8	0.196	0.209	0.239	0.205	0.268	0.452	0.225		*	NS	*	*	NS	*	*	*	*
9	0.083	0.097	0.145	0.116	0.160	0.185	0.179	0.200		NS	*	*	*	*	*	*	*
10	0.127	0.126	0.135	0.099	0.156	0.275	0.117	0.127	0.048		*	*	*	*	*	*	
11	0.127	0.139	0.171	0.138	0.190	0.239	0.225	0.281	0.085	0.120		*	*	*	*	*	*
12	0.157	0.167	0.232	0.250	0.188	0.221	0.309	0.306	0.116	0.201	0.193		*	*	*	*	*
13	0.185	0.194	0.226	0.193	0.234	0.350	0.153	0.105	0.162	0.071	0.256	0.226		*	*	*	*
14	0.208	0.207	0.234	0.195	0.230	0.362	0.224	0.264	0.148	0.112	0.223	0.257	0.141		*	*	*
15	0.065	0.099	0.064	0.115	0.084	0.117	0.109	0.204	0.060	0.100	0.121	0.139	0.167	0.127		*	NS
16	0.149	0.138	0.150	0.130	0.123	0.290	0.169	0.150	0.143	0.100	0.235	0.180	0.086	0.111	0.108		*
17	0.071	0.083	0.047	0.092	0.068	0.180	0.078	0.141	0.092	0.047	0.136	0.182	0.118	0.115	0.028	0.071	

Appendix 4: Figures

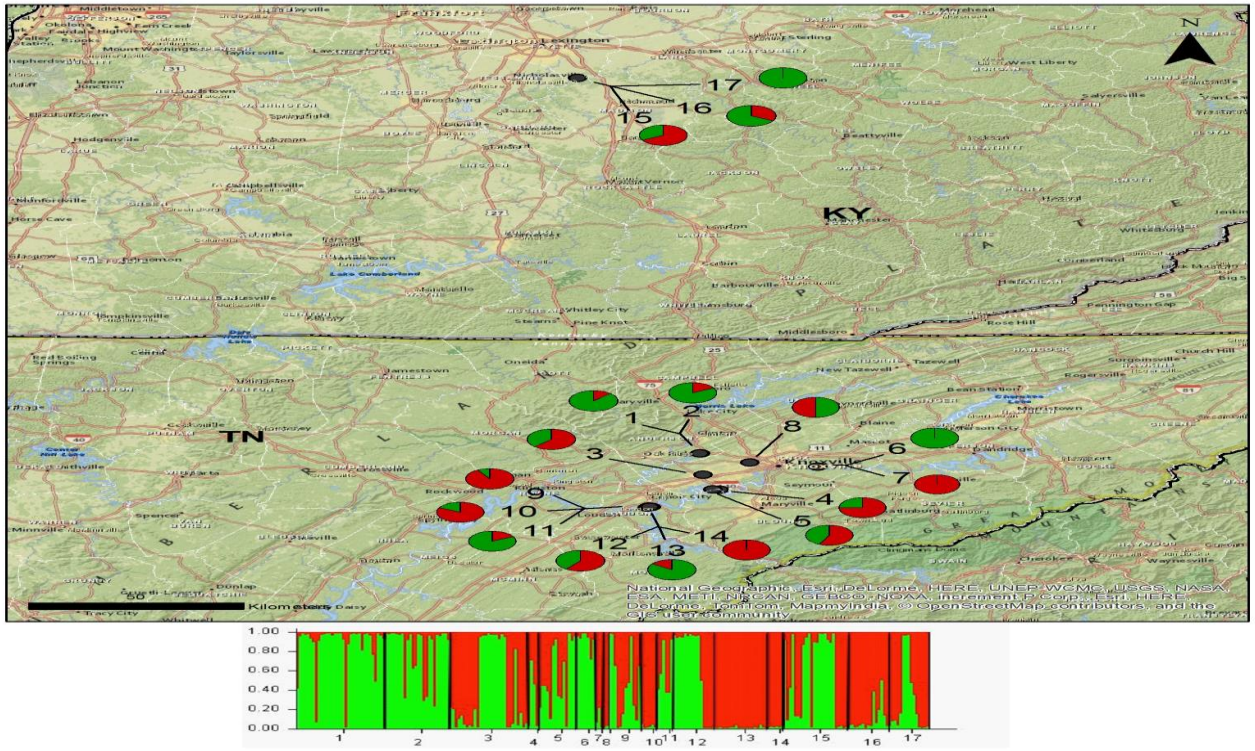

Figure 3-1. Locations in Kentucky and Tennessee of *Viburnum rufidulum* populations sampled for characterizations. Pie charts and bar plots represent the proportions of cluster membership (K = 2) of all individuals sampled, which were obtained with STRUCTURE 2.3.3 using the admixture model based on seven microsatellite loci and *V. rufidulum* collected from 17 sites Bar plots represent each individual's percentage of assignment to each of the two populations based on Bayesian assignment probabilities.

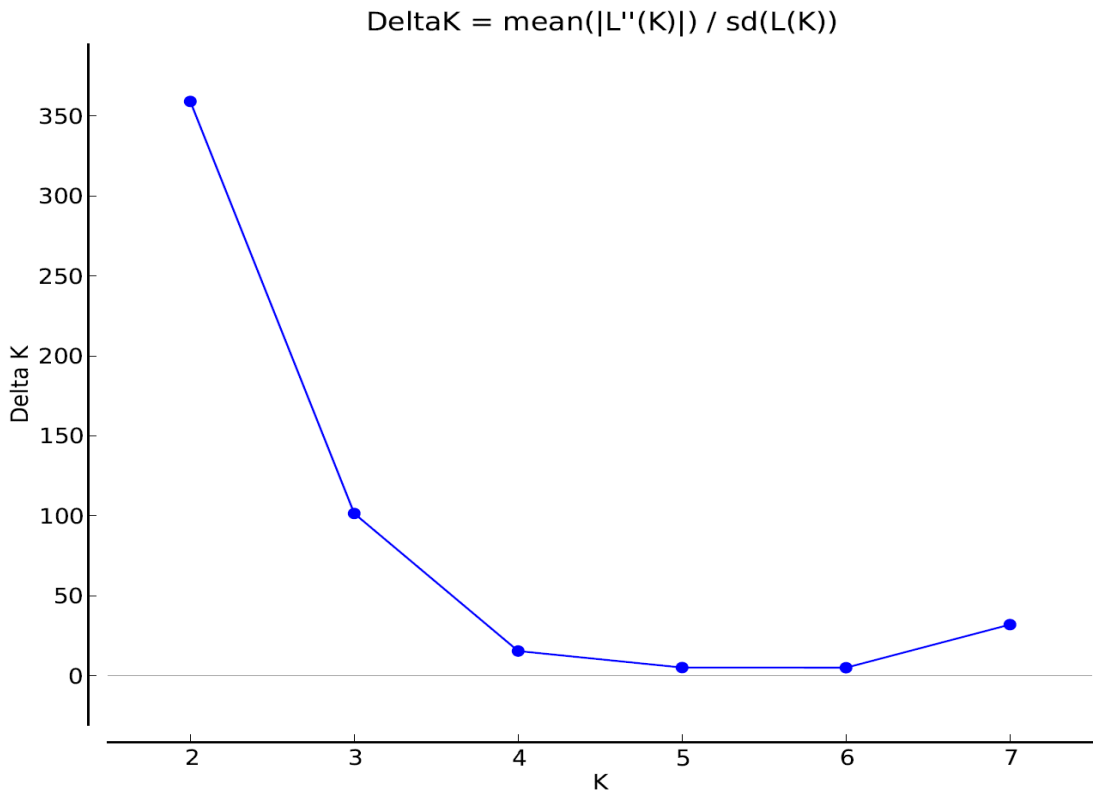

Figure 3-2. The most probable value of K (K = number of clusters) for 17 populations of *Viburnum rufidulum*, as determined by the program STRUCTURE 2.3.3, representing the number of populations as two with the ad hoc statistic, Δk, Evanno et al. (2005).

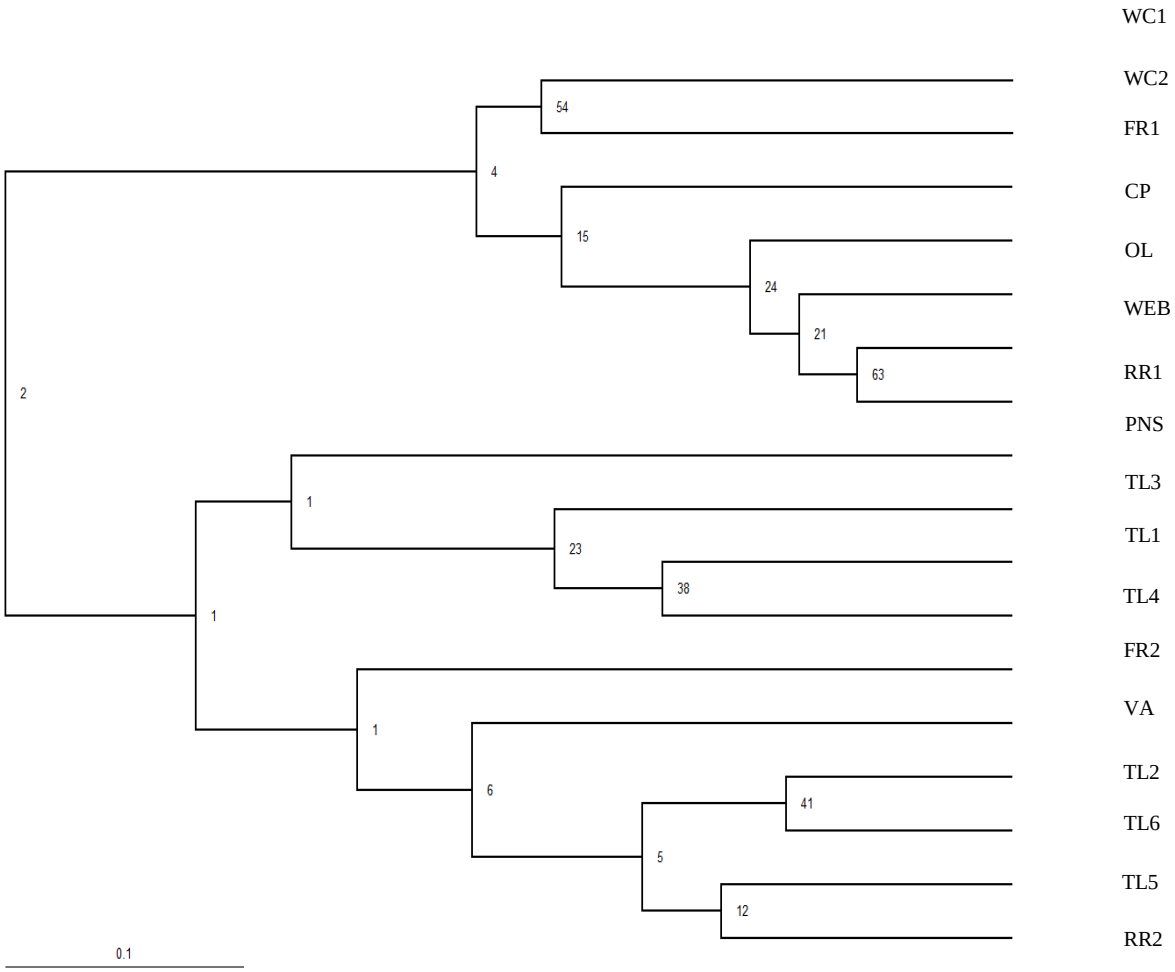

Figure 3-3. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram of the *Viburnum rufidulum* populations based on seven microsatellite loci generated from Populations 1.2.31 (Langella, 2002) including bootstrap support values. The dendrogram was displayed in TreeView (Page, 1996)**.** Five hundred bootstrap randomizations are reported as percentages at each node. The scale bar represents 0.1 substitutions per nucleotide site.

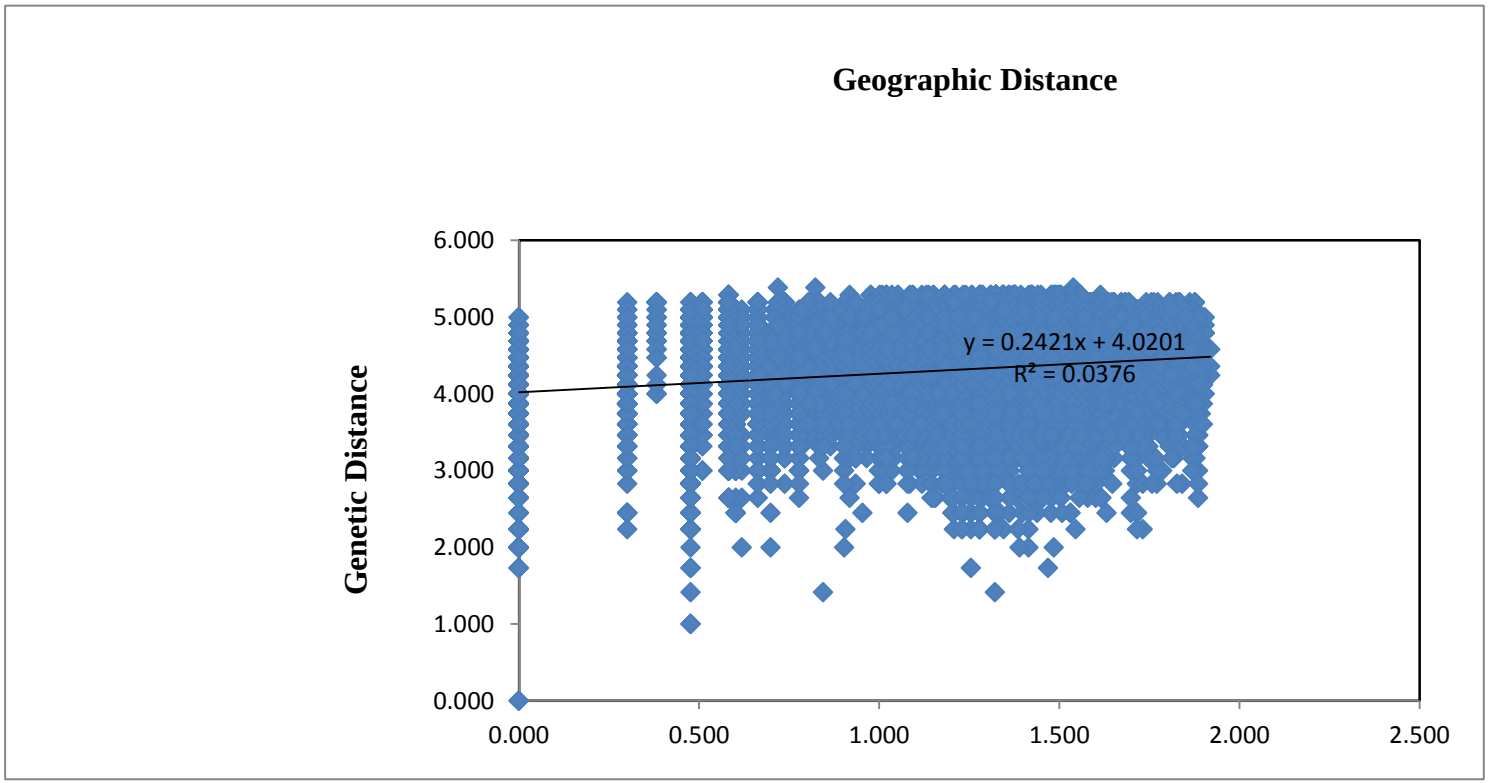

Figure 3-4. Pairwise genetic distance and geographical distances of 235 *Viburnum rufidulum* samples collected from 17 populations in Tennessee and Kentucky displayed as a scatterplot. The P value was obtained with the Mantel test after 9999 permutations using the program GenAlEx 6.5.

Chapter 4. Can phylogeny be determined from cross transferability of loci from three species in the genus *Viburnum*?

Abstract

Viburnum is a member of the Adoxaceae and is a large genus of woody shrubs consisting of approximately 160 species. In the past the genus *Viburnum* has undergone various reclassifications based on morphological and molecular studies. In this study 50 Viburnum species and five related genera were sampled. Thirty-three microsatellite markers were developed from *V. dilatatum, V. farreri*, and *V. rufidulum* and were utilized to characterize and assess the relationships between those various taxa. Cross transferability of the loci was analyzed and the number of alleles per locus ranged from 4 to 39. The polymorphic information content ranged from 0.42 to 0.97%. Three genomic microsatellite loci were used to construct a phylogenetic tree. The sequence alignments indicated well preserved primer sites throughout all the taxa analyzed, and resulted in successful cross transferability among many *Viburnum* species as well as limited amplification to other genera. We expect this set of SSR markers to have utility in future genetic studies, marker assisted selection, and breeding programs. We conclude that phylogenetic construction and interpretation with the use of SSR markers be completed with prudence.

Introduction

Viburnum species grow throughout the temperate and subtropical regions of the Northern Hemisphere (Hoch, 1995). There are approximately 20 North American species, 60 species from South and Central America, 4 European species, 30 North African species, and more than half of all species are indigenous to Asia (Dirr, 2007). Major centers of diversity occur in Asia and Central and South America (Morton, 1933; Donoghue, 1983). *Viburnum* is a genus of shrubs and small trees in the Adoxaceae,

which also includes the *Adoxa*, *Sambucus*, and *Sinadoxa* . It is the largest genus in the Adoxaceae with approximately 160 species (Winkworth and Donoghue, 2005) and was formerly a member of the Caprifoliaceae (APG II, 2003). While this genus has little variety in fruit and flower morphology, it is diverse in many other traits such as: fruit color, bud and inflorescence form and endocarp shape (Winkworth and Donoghue, 2005).

Viburnum was established in 1753 by Linnaeus and from that time the genus has undergone myriad treatments, sectional revisions as well as the placement into Adoxaceae from the Capriloliaceae. Ten sections of *Viburnum* were established in 1983 based on the lineage of the species placed in each of those clades (Hara, 1983). The sections were increased to 12 based on chloroplast and nuclear loci (Winkworth and Donoghue, 2005). The biogeographical analyses described five movements of *Viburnum* from the Old to New World by means of Beringia (Winkworth and Donoghue, 2005; Clement and Donoghue, 2011). In 2011, additional markers and samples redefined the clades and suggested that the sectional group Megalotinus be eliminated and that the superclades be expanded to include four new clades (Clement and Donoghue, 2011). Therein , Clement and Donoghue (2011) placed the former members of Megalotinus into the following four new suggested clades: Punctata, Lutescentia, Sambucina, and Coriacea. It is clear that this large genus will continue to undergo changes as molecular work advances (Dirr, 2007).

For the purpose of identifying plants and discerning genetic differences between related species, the Plant Working Group of the Consortium for Barcodes of Life recommended using the chloroplast coding genes *matK* and *rbcL* as a standard for plant

DNA barcoding (Plant Working Group, 2009). Moreover, the group suggested an additional list of noncoding plastid region loci where further discrimination was required. In a study of the species included in *Viburnum*, Clement and Donoghue (2012) attempted to increase the discriminating power of the *matK* and *rbcL* genes by combining them with the supplementary *trnG-psbA* and nrITS loci (Plant Working Group, 2009). However, studies were not successful in distinguishing between species using those genes (Pettengill and Neel, 2010; Wang et al., 2010). Additionally, the *matK* and *rbcL* loci could only differentiate 53% of the 112 *Viburnum* species tested (Clement and Donoghue, 2012). The authors reported that the discriminatory power of *matK* and *rbcL* had been "overrated." Furthermore, closely related species within the same clade could not be differentiated, even though there were obvious morphological and geographical variations in the plants (Clement and Donoghue, 2012). Finally, these authors suggested that future studies include supplementary loci to improve the success in discerning those species that are closely related. Here, we attempt to ascertain the feasibility of using microsatellites to create a phylogeny of *Viburnum* based on SSR cross transferability among species in that genera.

Microsatellites, also referred to as simple sequence repeats (SSRs), are a class of co-dominant markers. SSRs are tandemly repeating mono-, di-, to penta-nucleotide segments of DNA that occur throughout the genomes of most eukaryotic species (Powell et al., 1996). The number of repeats occurring at these loci varies greatly due to the high rate of mutation that may occur during DNA replication when polymerase slippage occurs (Bruford and Wayne, 1993; Peakall et al., 1998). Polymorphisms of the microsatellite loci are alternate forms of a gene, and can be detected easily via PCR

(Powell et al., 1996; Zhao et al., 2008). Conservation of the SSR unique flanking region allows for the design of primers that will amplify the microsatellite loci during PCR (Mace and Godwin, 2002).

The high mutation rate in the repeat motif of SSRs may make them a valuable supplement to discerning closely related species. The high variability of microsatellite loci offers great statistical power in discriminating between different species in the same genus (Gupta and Varshney, 2000; Hedrick, 2001). However, the regions flanking the SSR motif are also valuable for genetic studies. Species that are more genetically similar exhibit conservation of the microsatellite flanking regions and therefore facilitate cross transferability of primers (Arnold et al., 2002). Cross-species and genera transfer of SSR primer sites have been demonstrated in other taxa such as *Cercis* (Wadl et al. 2012), *Festuca* and *Lolium* (Elazreg et al. 2011), and a subset of *Viburnum* (Dean et al., 2011).

There is much debate and controversy as to whether SSRs have utility in creating phylogenetic trees. Although SSR markers have great versatility in population genetics, they are not considered the best tools for phylogenetic studies (Rossetto et al. 2002; Calonje, M. et al. 2009; van Zijll de Jong et al. 2011). The primary detractors are size homoplasy and due to high mutation rates, the question of whether SSRs are accurate molecular clocks (Calonje et al., 2009; Sun et al., 2009). Size homoplasy occurs when alleles are identical in state (in length or number of base pairs), but not identical by descent (Orti, G., et al. 1997; Doyle, J. et al, 1998). If non-homologous fragments co-migrate and are not recognized or controlled, the paralogous alleles will create erroneous phylogenies. In this case, the alleles appear to have similarity in the phylogeny, but in fact have different sequence motifs (Barkey et al., 2009). Homoplasy can occur within

the repeat motif region or the flanking region of SSRs (Calonje et al., 2009). In SSR studies, homology, which is similarity between characters due to a shared ancestry, can easily be confused with homoplasy. This can potentially lead to incorrect and inconsistent phylogeny construction as well as erroneous measures of relatedness, interpretation of population structure, and genetic diversity (Grimaldi and Crouau-Roy 1997; Barkey et al., 2009). Calonje et al. (2009) suggested that all orthologues undergo sequencing to ensure correct inferences of phylogeny.

Mutations within SSR repeat motif regions happen during replication when slip-stranding mispairing can occur and create new alleles (generally a greater number of base pairs) and may lead to homoplasy (Orti al., 1997). These mutations create SSR length polymorphisms, which make these markers valuable for intraspecific studies (Weber and May, 1989). However, SSRs typically have much higher mutation rates than other non-coding nuclear markers, making it challenging to get resolution between more distantly related taxa (Calonje et al. 2009). For a molecular marker to provide accurate historical inferences, mutations must be accumulated in a predictable manner (Sun et al., 2009). This brings the utility of SSR loci in creating phylogenies into question.

The relationship between species in this genus is further confounded because it is possible for members of one species to hybridize with other *Viburnum* species (Egolf, 1962; Clement and Donoghue, 2012). Hybridization coupled with the high rate of cross-species transferability of SSR markers suggests that much of the species in the genus may be very closely related and that some recognized taxa may in fact not be distinct species (Dean et al., 2011). Here, we utilized microsatellite markers in an attempt to characterize and distinguish species within the *Viburnum* genus. We

reviewed the findings of the Winkworth and Donoghue (2005) phylogeny created from a combined data set of chloroplast and nuclear markers. From that combined analysis, we choose *V. dilatatum* (section OW Odontotinus*), V. rufidulum* (section Lentago) and *V. farreri* (section Solenotinus) as representatives of the genus. We have previously developed *V. dilatatum* and *V. rufidulum* SSR markers, which we utilized in our current analysis (Dean et al., 2011; Dean et al., 2014). The objectives of our study were the following: 1) to develop a microsatellite library for *V. farreri*; 2) to determine relationships between various *Viburnum* species and other select taxa not in the genus using microsatellites from three *Viburnum* species libraries; 3) to ascertain cross-transferability of the SSR markers to species in other genera within the Adoxiaceae and the Caprofoliaceae; and 4) to construct a phylogeny of *Viburnum* species from microsatellite markers.

Materials and Methods

A genomic SSR library of *V. farreri* was developed as the third representative of the genus *Viburnum* based on a previous phylogenic analysis (Winkworth and Donoghue, 2005). Genomic DNA was extracted from unopened leaf buds of 12 *V. farreri* specimens. The quality of DNA was assessed a 2% agarose gel stained with ethidium bromide and images were recorded using the 2000 Gel Doc System (Bio-Rad Laboratories, Hercules, CA, USA). A small insert genomic DNA library was enriched for $(CA)_n$ simple sequence repeats (SSR) using a method developed by Wang et al. (2007). Briefly, 2.5 µg of genomic DNA from *V. farreri*, accession #1F collected in North Carolina was digested using the following restriction enzymes: AluI, HaeIII, RsaI, and StuI (0.5 U/ µl of each enzyme). The digestion was completed in three min at room

temperature (21°C), which produced blunt-ended fragments that ranged in size from 250 to 800 bp when visualized on a 2% agarose gel stained with ethidium bromide. Restriction enzymes were heat inactivated at 70° C for 20 min. The digested fragments were ligated to the SNX linkers: 5' CTAAGGCCTTGCTAGCAGAAGC 3' and 3' AAAAGATTCCGGAACGATCGTCTTCGp 5' using PCR conditions described by Hamilton et al. (1999). The ligated PCR products were purified by column (QIAquick PCR purification Kit, Valencia, CA, USA) and enriched with $(CA)_n$ when they were hybridized to $(GT)_{12}$ biotinylated oligonucleotides. Enriched $(CA)_n$ PCR products were captured utilizing the Streptavidin Magnesphere® Paramagnetic Particles (Promega, Madison, WI, USA) (Hamilton et al., 1999; Wang et al., 2007). The enriched PCR products were ligated to the *Eco*R V-cut pBluescript SK II (+) vector, (Stratagene, Hanover, MD, USA) using an 8:1 DNA fragment to vector ratio, and used to transformation into *Escherichia coli* TOP 10 Electrocomp™ cells (Invitrogen, Carlsbad, CA) using the Gene PulserXcell® Electroporation System (Bio-Rad, Hercules, CA, USA). The putatively transformed bacteria and 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-gal) and β-D-galactoside (IPTG) were plated onto Luria-Bertani-ampicillin (LB) media, and incubated at 37° C overnight following a method by Sambrook et al., (1989). White colonies, which contained the putative transformed bacteria were selected and grown overnight in liquid LB media and ampicillin at 37°C. One µl of *Escherichia coli* cells developed from selected colonies containing viburnum genomic Following a method by Wang et al., 2007, one µl of the colony containing media was added to a 10 µl PCR reaction containing the following: 1× PCR gold Buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.4 U AmpliTaq Gold DNA polymerase (Applied

Biosystems, Foster City, CA, USA) and 0.25 mM each of the following three primers T3, T7, and $(GT)_{12}$ and sterile water. The thermal cycle conditions were as follows: one cycle at 96° C for 2 min, followed by 35 cycles of 94° C for 1 min, 55° C for 1 min, 72° C for 1 min and one cycle of 72° C for 1 min. The amplified PCR products were visualized under UV light on a 2% agarose gel stained with ethidium bromide and primers that amplified and produced a smear in the gel (indicative of multiple products) were regarded as probably containing a microsatellite insert Wang et al., 2007). Colonies with putative microsatellites were selected and duplicated in 100 µl of LB-ampicillin freezing medium and grown at 37°C overnight (Sambrook et al., 1989). Plasmid DNA from the colonies of interest were selected and isolated using a modified alkaline lysis method and was sequenced (ABI Big-Dye Version 3.1terminators) on a Model ABI 3730XL capillary electrophoresis DNA sequencer (Applied Biosystems, Foster City, CA, USA). After the inserts were sequenced, 30 primer pairs were designed using Primer 3.0 (Rozen and Skaletsky, 1998) using optimum parameters of 50 % GC content, oligonucleotide length = 20 base pairs, and an annealing temperature of 56º. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA). The primer pairs were optimized for PCR using genomic DNA from three *V. farreri,* samples including two 'Album' plants and one 'Nanum' cultivar. A 10 µl PCR amplification reaction that was comprised of the following: 4 ng of genomic DNA, 2.5 µM forward and reverse primers, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1× PCR gold Buffer, and 0.4 U AmpliTaq Gold DNA polymerase. The thermal cycler conditions were: 1 cycle of 94°C for 3 min, and 35 cycles of 94°C for 40 s, 58°C for 40 s, 72°C for 30 s, and 1 cycle of 72°C for 4 min. We sized the amplicons using the QIAxcel, Capillary Electrophoresis

System (Qiagen, Valencia, CA, USA). Of the 30 primer pairs, 14 microsatellite markers for *V. farreri* were optimized and developed (Table 3). These 14 primer pairs were utilized to amplify DNA from all 55 taxa included in this analysis.

Young leaves and flower buds were collected from a total of 55 accessions of which 50 were species of *Viburnum* and the remainder from closely related genera in the Adoxiaceae including *Adoxa moschatellina, Sambucus nigra, and S.canadensis,* and in the Caprifoliaceae, including *Lonicera japonica* and *Weigela florida* (Table 2). Genomic DNA was extracted from all specimens using the QiagenDNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA). The DNA was quantified and evaluated using the Nanodrop® ND-1000 Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA from 55 accessions was amplified using 11 primers from *V. dilatatum,* 8 from *V. rufidulum* (Dean et al., 2011; Dean et al., 2014) and the 14 developed from *V. farreri* (Table 3).

We evaluated the interspecific cross transferability of genomic microsatellite markers from *Viburnum* as well as to other closely related genera. Eleven *V. dilatatum* (Dean et al., 2011), eight *V. rufidulum* (Dean et al., 2014) and fourteen *V. farreri* were used to amplify DNA samples of 50 *Viburnum* species. Additionally, one DNA sample each of *A. moschatellina, S. nigra, S.canadensis, L. japonica* and *W. florida* of the Caprifoliaceae were analyzed (Table 2). Amplification and sizing of the amplicons was performed using the same methods as described previously.

Three primers that amplified products in most of the taxa were selected for sequencing and phylogenetic studies (Table 1). DNA amplification of the three selected SSRs was completed as before except that 24 ng of genomic DNA was used in a 30 µl

reaction mixture that of the following: 2.5 µM forward and reverse primers, 2.0 mM MgCl2, 0.2 mM dNTPs, 1× PCR gold Buffer, 5% dimethyl sulfoxide, 0.4 U AmpliTaq Gold DNA polymerase, and sterile water. The thermal cycler conditions were as previously stated. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Purified PCR products were cloned using the p-Gem®-T Easy Vector System II (Promega, Madison, WI, USA) following the manufacture's guidelines except the ligation reactions were incubated for 19 h instead of 1.5 h at 37°C shaken at ~150rpm. The extended incubation period enhanced the number of both white and blue colonies formed on LB medium. White colonies were selected to inoculate the following PCR reaction mixture for colony screening: 2.5 µM T7 and SP6 primers, 2.0 mM MgCl2, 0.2 mM dNTPs, 1× PCR gold Buffer, 5% dimethyl sulfoxide, 0.4 U AmpliTaq Gold DNA polymerase and sterile water to bring to 10 µl. The PCR conditions were as above. The amplified PCR products were visualized under UV light on a 2% agarose gel stained with ethidium bromide and bands near the expected size range were selected. Five to ten clones were selected and sequenced for each accession to control for polymorphisms. The clones were then screened and sequenced in reverse and forward direction using the T3 and T7 PCR primers on the Model ABI 3730XL capillary electrophoresis DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Data Analysis

The number of *V. farreri* alleles (A) and polymorphic information content (PIC) were calculated using the program CERVUS 3.0 (Kalinowski et al., 2007). PIC is described by Botstein et al. (1980) using the formula: PIC = 1- $\sum P_i^{2,}$ here p_i is the

frequency of the *i*th allele for each of the SSR markers. In addition, the expected (H_e), and observed (H_o), heterozygosity were also calculated with the same program.

Summary statistics for the complete set of 33 loci were also analyzed in CERVUS 3.0 as described (Kalinowski et al., 2007) (Table 3). The base pair sizes from the 33 markers and 55 samples were coded into a data matrix with 9 representing missing or null alleles. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram (with 500 bootstrap analysis randomizations) was generated to examine species and genera relatedness genetic distance matrices based on Nei et al. (1972) using the program POPULATIONS v. 1.2.30 (Langella, 2002) (data not shown). The results were generated, viewed, and edited using TREEVIEW 1.6.6 software (Page, 1996). The initial dendrogram included all 55 accessions, however the results did not cluster into meaningful groups. Therefore citing *Vitis and Actinidia*, fewer species studies samples were used due to other studies having success creating phylogenies with a limited number of taxa (Rossetto et al., 2002; Korkovelos et al., 2008). A principal coordinate analysis (PCoA) was performed in GenAlEx version 6.5 (Peakall and Smouse, 2006) to analyze the relationships among the various genotypes.

Three primers (VF004, VF005 and VF016) that amplified and demonstrated cross-transferability across almost all 55 accessions underwent sequencing of forward and reverse strands (Table 1). Discrepancies in base pairs from independent sequences were reconciled and aligned using Sequencher 5.2 Gene Codes, Ann Arbor, MI, USA. Sequences were aligned with CLUSTALX version 2.1 (Thompson et al., 1997) and Bioedit (Hall, 1999) was also used to assist in manual alignment to mitigate gap locations between the sequences.

For phylogenic analysis a separate data matrix and tree was prepared and generated for each of the three microsatellite primers and the taxa using the program Seaview 4.5.2 (Gouy et al., 2010). Samples that did not amplify for any of the three SSR loci were coded as missing data using the program Bioedit (Hall, 1999) and a text editor. The phylogenetic trees were calculated with the neighbor joining method using 1000 replicates, employing the ignoring all gap sites option and Kimura's evolutionary distance model (Kimura, 1980). Some samples were not included in order to create a more global consensus and the final data set consisted of 22 taxa and was rooted with *A. moschatellina*. Tree data sets were then concatenated into a supertree with Seaview 4.5.2 and clade robustness was measured using 1000 replicates using the same parameters previously mentioned.

A *Viburnum* phylogenetic tree was rooted with *S. canadensis* by Winkworth and Donoghue in a previous study (2005). When we attempted to include *S. canadensis*, but it was double in size in base pair length compared to the other taxa it was being aligning with, and made the alignment problematic. Another study had similar problems rooting *Sambucus* with *Viburnum* due to the vast genetic distance between the two genera and therefore used *V. clemensiae* as a sister to *Viburnum* (Clement and Donoghue, 2011). A sample of *V. clemensiae* was not available, therefore, *A. moschatellina* was used as an outgroup.

Results

The *V. farreri* SSR library produced a total of 296 (average of 21) alleles across the 14 loci. The number of alleles per locus ranged from four (VF030) to thirty-nine (VF039). Observed heterozygosity values for the total group of VF markers were 0 to

0.83 and H_e ranged from 0.69 to 0.98. The markers did not conform to Hardy-Weinberg equilibrium as all values of H_o were lower than the H_e values. The lowest PIC value was found at loci VF 030 (0.63), and VF002 exhibited the highest PIC value of 0.97.

Thirty-three *Viburnum* SSR primers were designed and utilized to assess their ability to amplify in fifty species of *Viburnum* and four accessions of related genera (Table 1). Amplification to other *Viburnum* species ranged from 9 species (VD012) to 50 different species (VF001, VF004, and VF005 each) (Table 3). The 14 primers developed from the *V. farreri* library boasted the greatest occurrence of cross amplification to other *Viburnum* species (42-50) with a mean of 40 different species. While the 8 primers of the *V. rufidulum* library cross amplified 12 to 40 species with a mean of 25 *Viburnum* species, the 11 primers designed from *V. dilatatum* cross amplified an average of 29 different *Viburnum* species. Of the total group of primers, seven (VF001, VF009, VF013, VF016, VF029, VD016, and VR044) cross amplified in *A. moschatellina*, and six (VF004, VF014, VF016, VF029, VD016, and VR044) in *L. japonica*. A total of five markers amplified in samples of *Sambucus*: five (VF002, VF004, VF 014, VF029, and VR011) across *S. canadensis*, and three each in *S. nigra* (VF002, VF 014, and VF029). Three of the SSR loci cross amplified in *W. florida* (VF016, VF029, and VR044). VF001, VF004, and VF005 were able to amplify to 100% of the various *Viburnum* species in this study. Primer VF029 was the only one that was able to cross amplify across species in all five genera.

The number of alleles ranged from four (VD006 and VF030) to 39 (VF 002) (Table 3). Three of the SSR loci were found to be monomorphic: VD006, VF030, and VR043 (Table 3). Thirty of the SSR loci were polymorphic. Primer VF004 produced

amplification products in all of the *Viburnum* species and was polymorphic in 45 *Viburnum* species. The greatest degree of gene diversity and polymorphism found in the *V. dilatatum* and *V. rufidulum* loci were at loci VD014 (H_e=0.96, PIC=0.94) and VR 028 (H_e=0.97, PIC=0.96); the least was detected at loci VD006 (H_e=0.47 PIC=0.42) and VR012 (H_e=0.75 PIC=0.78). The greatest PIC value was VF002 (0.97%).

Loci selected to be sequenced were based on the percent of total amplifications, PIC value and the primer's ability to amplify across different genera, as well as other species of *Viburnum* (Table 2). Primers designed from the *V. farreri* libraries met those criteria. Primers VF004 (% amplification 0.98, PIC 0.90, H_e 0.90), VF 005 (% amplification 0.93, PIC 0.95, H_e 0.96), and VF016, (% amplification 0.94, PIC 0.85, H_e 0.87), were selected. The priming regions of all three markers were highly conserved.

The phylogenic analysis for the concatenated data set revealed a distinct structure however the tree was not very robust (Fig. 1). The nodes with boot strap values greater than 50% were the following: *V. lentago* and *V. prunifolium*; *V. recognitum* and *V. ellipticum; V. cylindricum* and *V. phlebotrichum*; and *V. farreri* and *V. erosum*. The 22 taxa were clustered into three main groups with *A. moschatellina* as outgroup. Clade one: *V. lentago. V. prunifolium, V. plicatum, S. nigra, V. acerfolium, V. recognitum* and *V. ellipticum.* Clade two: *V. propinquum, V. carlesii, V. molle, V. dentatum, V. rufidulum, V. nudum, V. dilatatum, V. cylindricum,* and *V. phlebotrichum.* The third clade was comprised of the following species: *V. odoratissimum, V. farreri, V. erosum, V. erubescens* and *V. japonicum.*

The genotypes were grouped according to a principal coordinate analysis (Fig. 2), that indicated that the first, second, and third principal coordinate explained 12.04%, 8.67%, and 7.56% (~30%) of the total variation, respectively.

Discussion

The set of *V. farreri* SSR loci had an average PIC of 0.85. Out of a possible total of 700 amplifications, 339 or ~48% produced polymorphic amplicons. These results were similar to a review of interspecific transferability of SSR markers by Barbará et al. (2007), in which eudicots the % of polymorphism was 48 ± 4. The only markers that produced polymorphic products in other genera were VF002 (in *S. nigra*) and VF014 (in *S. canadensis*). This library also displayed the greatest intergeneric cross transferability, which will be discussed.

Sequence data from three nuclear, nrITS (nuclear ribosomal internal transcribed spacer), *WAXY1* and *WAXY2* and two chloroplast loci, *trnK* intron and *psbA-trnH* IGS (intergenic spacer) were combined into a data set to characterize the genus *Viburnum* (Winkworth and Donoghue, 2005). In that study the authors were able to successfully resolve polytomies within their individual data set phylogenies that occurred within five of the sections: OW Odontontinus, Oreinotinus, Opulus, Viburnum and Solenotinus. By combining the chloroplast and nuclear gene data sets they were able to resolve the following species relationships with usually greater that 90% bootstrap values: *V. acerifolium, V. kansuense, V. dilatatum, V. japonicum, V. erosum, V. lobophyllum,* and *V. cylindricum* (OW Odontotinus); *V. stenocalyx, V.* hartwegii, *V. jucundum, V. triphyllum* and *V. dentatum* (Oreinotinus); *V. edule, V. sargentii,* and *V. trilobum* (Opulus); *V. erubescens, V. farreri, V. odoratissiumum, V. sieboldii*, and *V. suspensum* (Solenotinus*);*

and *V. carlesii, V. lantana* and *V. utile* (Viburnum) (Winkworth and Donoghue, 2005). We attempted to characterize as many species of *Viburnum* that were included in the former study and were able to include 29 species represented by Winkworth and Donoghue (2005). Additionally, the decision on which species to select for library construction was based on consideration of which species would give a broad representation of the entire genus. Those libraries were therefore developed from *V. dilatatum, V. farreri,* and *V. rufidulum.*

The cross transferability of SSR loci was analyzed with *V. dilatatum, V. farreri,* and *V. rufidulum* in 50 *Viburnum* species and five other related genera. Thirty- three SSR loci from three *Viburnum* species exhibited an average cross species amplification of 63%. In addition, seven or 21% of the loci were able to demonstrate intergeneric transferability. And although cross transferability to other genera was much lower, this study validates the ability of SSR markers to be employed across different genera as well as across different plant families (Adoxaceae and Caprifoliaceae).

We found interspecific cross transferability was considerably lower when compared to a study another woody ornamental-*Cercis canadensis*. In the *Cercis* SSR marker study, the cross species amplification rate was 95% (Wadl et al., 2012). Although that study included 50 *Cercis* species cultivars, it was comprised of only eight species, considerably less than the 50 species of *Viburnum* analyzed in this study. Additionally, 68 *Cercis* SSR loci were employed, all of which were polymorphic (Wadl et al., 2012). The lower cross species amplification (63%) observed in this study might be due to the limited number of markers used. This illustrates the importance of employing large numbers of SSRs in molecular studies.

Cross genera amplification of the *Viburnum* SSR markers occurred in *A. moschatellina, S. canadensis, S. nigra, L. japonica* and *W. florida.* Interestingly, *A. moschatellina* is distinguished from those other members of Adoxaceae and Caprifoliaceae by its unique morphological traits. Unlike the tree and shrub form of *Viburnum, Lonicera, Sambucus,* and *Weigela- Adoxa* is a perennial herb that reaches a height of only 5-20 cm (http://plants.usda.gov/core/profile?symbol=ADMO). Although *Viburnum* is morphologically more similar to *Sambucus,* these SSR markers transfer as readily to *A. moschatellina* and *L. japonica*. *Viburnum* was formerly placed in the honeysuckle family (Caprifoliaceae), which may explain why the SSR loci were able to amplify in *L. japonica* as well as *W. florida*. However the frequency of intergeneric cross transferability (21%) of our markers was much lower than found in a recent study (71%) (Rai et al., 2013). There, the high rate of transferability of a set of guava SSR markers to genera in Myrtoideae (a large family of woody plants) was attributed to the family being closely related (Rai et al., 2013). Our findings provide additional evidence that Caprifoliaceae and the Adoxaceae are perhaps more distantly related, and supports the past movement of *Viburnum* and *Sambucus* from the Caprifoliaceae to Adoxaceae. While closely related species are expected to have conserved priming sites, this conservation of priming sites is less common in more distantly related species (Ekue et al., 2009). This study offers evidence of modest cross transferability of SSR loci across the genus *Viburnum* to other genera within Dipsacales, as well as high rates of interspecific cross transferability. Additionally, the average PIC and number of alleles of this set of 33 SSR markers are 0.83 and 17 (VF library loci having the highest of both values) indicating that they will be informative in future studies.

Although microsatellite markers have been extensively utilized to study genetic relationships at the interspecfic level, their utility has been criticized in the use of inferring phylogenic relationships (Calonje et al., 2009). Issues that may limit accurate phylogeny construction include the following: poor cross transferability, homoplasy, and rapid mutation rates of the SSR motif regions (Bruford and Wayne, 1993; Takezake and Neir, 1996; and Calonje, et al., 2009). The total variation of length of SSR sequences arise as a result of insertion and deletions and single nucleotide polymorphisms that occur within the SSR region, and in the flanking regions as well (Calonje et al., 2009). This set of SSR markers demonstrated the ability to cross transfer interspecifically, and we next tested their merit in phylogenetic tree construction.

Previous studies have found that combining sets of data increases discriminatory power and can produce a more robust tree (Devulder et al., 2005; Winkworth and Donoghue, 2005; Weisrock et al., 2012). Conversely, the assembly of multiple data set trees can result in strongly supported, but erroneous phylogenies (Kubatko and Degnan, 2007; Weisrock et al., 2012). Therefore the selection of gene/loci is important. Amplified sequences from the VF004, VF005 and VF016 markers were used to generate individual trees prior to concatenating the data set. We evaluated the global compatibility of the three trees, for consensus is considered desirable when creating phylogenetic trees (Steel et al., 2000; Devulder et al., 2005; Weisrock et al, 2012). The three trees shared a modest amount of global compatibility (data not shown). Incongruity between phylogenies is a prevalent problem when comparing individual loci to each other (Rokas et al. 2003). Lack of concordance can be attributing to sampling error, different branching histories, and differing stochastic processes that affect characters (de Queiroz

et al., 1995). Both empirical and simulation studies have concluded that gene trees with high levels of discordance produced erroneous phylogenetic trees (Kubatco and Degnan, 2007; Belfiore et al., 2008; Weisrock et al., 2012). Donoghue (2006) also reported incongruence in data while analyzing *Viburnum* with nuclear and chloroplast markers, which lead to the identification of hybrid species. An example is *V. prunifolium*, a probable hybrid species of *V. lentago* and *V. rufidulum* (Donoghue, 2006). Interestingly, *V. rufidulum* failed to group with *V. lentago* and *V. prunifolium* in our analysis suggesting that SSR markers are also susceptible to discordance in phylogeny construction when hybrids are involved.

We opted to follow methods in studies that were successful using SSR markers to construct phylogenies. In attempt to circumvent discordance in our data we: reduced the number of taxa used to generate more global consensus between the three trees, included representatives from the various *Viburnum* clades, selected the allele that was closest to the expected base size of each SSR loci for each sample and, evaluated whether to include or exclude the flanking regions of the SSRs.

A previous study found the resolving power of microsatellite markers in phylogenies is limited to the number of taxa represented (Rossetto et al., 2002). Therefore our data set was reduced from 55 to 22 accessions in an attempt to generate more global consensus between the three trees. We included representatives from the various *Viburnum* sections and taxa from other genera as well.

An often unconsidered aspect in gene concatenation is which allele to include in nuclear data sets (Weisrock et al., 2012). Heterozygous alleles can complicate the concatenation of genes while creating phylogenies and affect outcomes. Randomly

selecting an allele does not necessarily obviate this problem (Weisrock et al., 2012). For the scope of this study the allele that was closest to the expected base pair size for that particular locus was included in the data set.

Furthermore, on close examination of the sequences size homoplasy was present. For example in the phylogeny created by VF005, *V. rafinequianum—V. ellipticum* were identical. While these two samples where identical in length there were sequence variations in the flanking regions. For this study, if homplasy was present in a pair of samples, one sample was randomly excluded. A major problem in attempting to construct the phylogeny is the uncertainty in knowing whether the loci used were orthologous or not. Rossett et al. (2002) successfully resolved a phylogeny of eight species from four genera using only three SSR markers which were located in the coding regions of the *Vitis* genome (Calonje et al., 2009). When a BLAST search was performed with the *V. farreri* loci used in our study no similarities were found, so it is not known if this set of markers are situated in coding or non-coding regions or whether these loci have a function.

In the study of *V. vinifera* the authors were able to successfully resolve the phylogeny of eight closely related species by removing the SSR regions and using only the flanking regions (Rossetto, M. at el., 2002). The authors based their decision on the following issues: the presence of homoplasy in SSRs, lack of a validated evolutionary clock (due to the rapid mutation rate of SSRs (Goldstein et al., 1995), and resolution between recently diverged species tends to be low (Rossetto,at al., 2002). Their results were more informative than that of a former study that used a combined analysis of *trnL* intron and ITS1. Rossetto, et al. (2002) concluded with a caveat that the resolution power

of their study was patently limited to the small number of samples employed. In addition, those SSR loci were located in the coding regions of the *Vitis* genome (Rossetto, M. at el., 2002; Calonje et al. 2009). When the SSR flanking regions were removed from our sequences (data not shown) and phylogenetic trees were constructed, the results provided even less concordance than when the SSR regions were included. When the SSR regions were removed in a study of citrus, the phylogenetic tree was much less resolved indicating the utility of those regions in deducing evolutionary relatedness in citrus accessions (Barkely et al., 2009). It appears that when concatenating multiple genes that larger data sets may benefit from the inclusion of the SSR regions, at least in the analysis of *Viburnum*.

The final phylogenic analysis for the three concatenated SSR loci and 22 taxa revealed a well-defined structure, but the tree was not very robust (Fig. 1). The only nodes that demonstrated boot strap values greater than 50% were the following: *V. lentago* and *V. prunifolium; V. recognitum* and *V. ellipticum; V. cylindricum* and *V. phlebotrichum*; and *V. farreri* and *V. erosum*. The 22 accessions were clustered into three main groups. One group included all North American species of *Viburnum* with the exception of *V. plicatum*, which is indigenous to Asia. A second cluster was comprised of both North American and Asian species. Finally, the third cluster included only Asian species. *Sambucus nigra* grouped *with V. plicatum* in the first cluster, two other subgroups consisted of: *V. lentago* and *V. prunifolium* and a third subgroup of *V. acerifolium, V. recognitum* and *V. ellipticum.* The second major clade was comprised of two subgroups. One with *V. nudum, V. dilatatum, V. cylindricum* and *V. phlebotrichum.* The second grouped *V. carlesii, V. molle, V. dentatum* and *V. rufidulum* together. Both of

these subgroups were of mixed Asian and North American species. *V. propinquum* was outside of these two subgroups. The third major clade grouped the Asian species *V. odoratissimum, V. farreri,* and *V. erosum* together and *V. erubescens* and *V. japonicum* were outside of this subgroup. Our combined data set shared modest consistency with phylogenetic trees in the previous study that examined combined chloroplast and nuclear loci (Winkworth and Donoghue, 2005). A major limitation of this study may have been using one individual to represent each taxa. Kubatka and Degnan (2007) found that sampling one individual per species to be a factor that can lead to poor results when concatenating data sets.

As we examined different tree topologies, *S. nigra,* which was not included in past Viburnum studies, consistently grouped with the *Viburnum,* where it was placed with *V. plicatum*. While this could be an artifact of a mislabeled sample, it also raises the question of whether the two are closely related genera that will continue to undergo further revisions in their classification.

The SSR data revealed a structure similar to current classifications, but overall the bootstrap values were low. However, regardless removed or adding samples to the analysis three clusters occurred. Three major subclades have also been revealed in former studies of Viburnum (Winkworth and Donoghue, 2004; Winkworth and Donoghue 2005). While there are some similarities such as *V. erubescens, V. farreri,* and *V. ordoratissimum* (Solenotinus) grouping together, *V. erosum* and *V. japonicum* (Odonotinus) also fall within this clade.

There was some agreement of the three clusters in the PCoA with the clades on the dendrogram, which also revealed 3 main sections. Interestingly there were patterns of

agreement with this study as well as that of Winkworth and Donoghue (2005). Two clusters grouped in agreement with Winkworth and Donoghue (2005): *V. dilatatum, V. erosum,* and *V. acerifolium; V. prunifolium* and *V. lentago* (Winkworth and Donoghue, 2005). There were three clusters that formed groups similar to the phylogeny of this current study: *V. phlebotrichum, V. rufidulum*, and *V. cylindricum; V. farreri, V. odoratissimum,* and *V. erubescens;* and *V. plicatum* and *S. nigra*. The remaining taxa did not disseminate into any meaningful groups.

In this study, we: characterized a novel *V. farreri* SSR library, utilized 33 *Viburnum* loci to characterize taxa and assess cross transferability in Adoxaceae and Capriloliaceae, and attempted to construct a *Viburnum* phylogeny. Due to the high rate of conservation of primer sites, this set of markers will be great utility in further intra and interspecific studies. In addition the cross amplification demonstrated by these loci may assist in guiding hybridization selections. In the future, resolution of phylogenies of the genus *Viburnum* using SSRs could benefit by including many more makers, and SSRs that occur in known coding regions as well sampling more than one individual to represent each species. In addition to the limited number of markers used in this study, the lack of congruence that was found in the individual (one loci) trees may have reduced our ability to resolve the concatenated tree. While this set of *Viburnum* markers did exhibit a modest cross transferability to other genera many did amplify in various *Viburnum* species. Although this set of loci did not provide resolution of the phylogenetic tree, we expect the markers to be of great utility for breeding programs and species and cultivar identification.

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Appendix 5: Tables

Table 4-1. Characteristics of *Viburnum farreri* microsatellite primers that were used to analyze cross transferability in samples of *Viburnum* and other closely related genera, including the Genbank accession numbers, primer sequence, repeat motif, expected size, number of alleles, and polymorphic information content (PIC).

Locus	Primer sequence (5'-3')	Repeat motif	Expected size (bp)	Number of alleles	PIC
VF004	F:GTGGTTGAGTATTGCAGGAAGG R:TCTACACACACCACACACAACC	GT_{12}	176	24	0.88
VF005	F:CCCAAATTGCTTATTCTTCTGC R:GCATTCTTTGAACCCTTTGG	TG_{14}	154	33	0.95
VF016	F:ACACACCACACACAACCACA R:AGTATTGCAGGAAGGCATGG	CA_{12}	164	20	0.85

Table 4-2. *Viburnum dilatatum*, *V. rufidulum* and *V. farreri* SSR loci cross-species transferability to selected taxa, and allele sizes.

	Allele sizes (bp) for loci											
Accession	VD003	VD004	VD005	VD006	VD009	VD012	VD014	VD016	VD017	VD018	VD019	VR004
V. erubescens	152:152	105:123	—	152:152	105:123	—	152:152	105:123	—	152:152	105:123	—
V. odoratissum	94:94	—	94:94	—	94:94	—	94:94	—	94:94	—	94:94	—
V. trilobum	114:125	—	114:125	—	114:125	—	114:125	—	114:125	—	114:125	—
V. sieboldii	85:153	113:122	—	—	—	—	165:165	—	—	—	—	176:176
V. sargentii	—	123:132	118:142	—	126:126	—	163:183	—	135:135	104:104	118:129	126:126
V. plicatum	83:83	107:107	—	—	—	—	147:186	—	—	102:102	—	174:174
V. rafinesquianum	107:112	104:109	138:138	—	138:138	—	—	—	149:149	102:102	139:139	194:194
V. carlesii 'Diana'	112:152	—	135:135	—	—	—	172:172	—	—	102:102	—	172:172
V. molle	105:105	104:109	142:142	—	—	—	—	81:81	—	104:104	132:132	192:192
V. lantana	114:114	—	133:133	—	—	—	165:165	—	—	104:104	—	174:192
V. ellipticum	107:107	114:114	133:142	—	—	—	—	80:80	—	104:104	150:150	192:192
V. dentatum 'Blue Muffin'	99:105	104:109	133:142	—	134:134	—	—	87:87	153:153	102:117	134:134	160:160
V. utile	112:112	111:111	109:109	—	111:126	125:125	167:167	—	—	102:102	123:123	180:180
V. tinus 'Compactum'	105:105	114:120	126:138	—	117:122	—	141:158	85:85	149:149	108:108	147:147	—
V. lentago	127:127	—	149:166	—	124:124	—	—	—	—	—	109:109	190:202
V. cinnamomifolium	98:98	—	—	—	118:140	—	143:158	85:85	145:145	102:102	105:114	184:184
V. prunifolium	131:131	—	126:126	—	—	—	—	—		—	102:102	109:109
V. lobophyllum	131:131	—	126:126	—	—	—	168:168	80:80	157:157	102:102	114:134	—
V. rufidulum	133:133	—	142:142	—	—	—	—	—	157:157	—	109:109	204:204
V. melanocarpum	99:99	104:104	135:135	130:130	140:140	125:125	163:172	81:81	157:157	104:104	125:152	—
V. nudum	109:116	111:111	117:117	—	145:145	—	240:240	—	1571:57	102:102	—	178:184
V. japonicum 'Variegatum'	137:137	105:105	140:171	130:130	144:144	—	168:168	81:81	159:159	106:106	125:125	—
V. foetidum	99:112	104:109	130:142	—	136:144	—	—	87:87	15715:7	102:102	130130:	160:160
V. propinquum	101:101	120:129	—	132:132	127:127	—	163:165		143:143	102:102	—	—
V. luzonicum	112:112	104:123	135:147	144:144	126:126	129:138	165:165	80:80	157:157	102:102	—	—
V. bracteatum 'Emerald Luster'	99:112	104:114	133:142	—	133:133	—	—	—	155:155	102:102	130:130	190:202
V. farreri 'Nanum'	155:155	145:145	131:131	—		—	140:140	—	—	—	102:102	—

Table 4-2. Continued.

Allele sizes (bp) for loci

Accession	VD003	VD004	VD005	VD006	VD009	VD012	VD014	VD016	VD017	VD018	VD019	VR004
V. rhytidophyllum 'Cree'	112:112	109:109	109:122	—	—	—	13414:1	—	—	102:102	—	182:182
V. phlebotricum	118:140	104:104	146:146	130:130	133:133	127:127	185:185	80:80	157:157	—	123:138	—
V. awabuki 'Chindo'	153:153	109:109	—	—	—	—	138:138	—	—	102:102	—	204:204
V. grandiflorum	150:153	131:131	188:188	—	—	—	150:156	—	—	104:104	—	—
V. ichangense	109:109	104:104	126:126	—	144:144	—	167:183	87:87	157:157	104:104	130:141	178:184
V. bitchiuense	114:114	105:105	115:124	—	—	—	167:167	—	—	104:104	125:125	—
V. burejaeticum	114:114	—	115:115	—	—	—	95:176	108:108	—	102:102	118:118	—
V. dasyantum	99:111	104:114	133:144	—	138:149	—	—	80:80	153:153	102:102	130:130	176:176
V. recognitum	99:105	104:109	142:142	—	—	—	—	—	—	—	—	—
V. wilsonii	118:118	102:111	162:162	—	—	—	176:176	81:81	157:157	102:102	121:121	—
V. acerifolium	—	105:105	153:153	—	122:122	127:127	154:154	—	155:155	102:102	118:118	—
V. dilatatum 'Asian Beauty'	109:109	105:120	147:168	128:128	111:118	131:148	145:183	108:108	157:157	102:102	134:134	—
V. erosum	—	—	—	130:130	—	109:109	156:156	93:93	157:157	102:102	123:123	—
V. davidii	—	114:114	—	—	117:117	—	152:168	83:83	145:145	104:104	105:116	—
Adoxa moschatellina	—	—	—	—	—	—	—	214:214	—	—	—	—
Sambucus canadensis	—	—	—	—	—	—	—	—	—	—	—	—
V. atrocyaneum	99:99	120:120	—	—	134:134	—	141:161	94:94	145:145	102:102	—	—
V. buddleifolium	114:114	—	109:109	—	—	—	134:150	—	—	—	—	—
V. hupehense	107:122	104:104	126:146	130:130	114:126	138:138	158:163	94:111	157:157	102:102	125:134	—
V. nervosum	114:114	—	—	—	—	—	149:165	—	—	—	—	178:192
V. opulous	103:103	104:122	107:107	130:130	126:126	—	172:180	—	—	100:100	114:114	—
V. schensianum	114:114	—	—	130:130	—	—	172:172	108:108	—	102:102	121:121	—
V. setigerum	111:129	104:104	155:155	130:130	126:138	103:127	176:176	108:108	157:170	102:102	138:138	—
V. suspensum	—	136:141	153:193	—	—	—	—	87:87	—	—	—	178:178
V. cylindricum	114:114	104:104	126:146	—	134:142	—	154:163	—	—	102:102	118:118	—
Loricera japonica	—	—	—	—	—	—	—	193:193	—	—	—	—
Weigela florida	—	—	—	—	—	—	—	—	—	—	—	—
Sambucus nigra	—	—	—	—	—	—	—	—	—	—	—	—

Table 4-2. Continued.

	Allele sizes (bp) for loci											
Accession	VR005	VR008	VR011	VR012	VR028	VR043	VR044	VF001	VF002	VF004	VF005	VF007
V. erubescens	180:180	115:115	178:178	250:250	—	212:212	153:153	331:331	257:281	229:229	197:197	287:305
V. odoratissum	202:202	115:115	176:176	250:250	148:148	—	169:169	332:332	271:271	230:290	189:189	312:312
V. trilobum	—	—	212:212	242:242	159:174	—	149:153	279:320	279:292	182:229	192:192	265:285
V. sieboldii	—	—	—	250:250	8484	173:173	153:153	329:329	260:260	232:232	192:192	276:294
V. sargentii	—	120:120	186:192	242:242	179:179	—	153:153	281:320	284:284	184:227	192:192	218:265
V. plicatum	—	—	—	—	244:244	—	151:151	327:327	297:297	229:229	182:182	293:293
V. rafinesquianum	135:135	—	160:160	—	105:233	—	153:153	279:314	305:305	182:227	193:210	267:285
V. carlesii 'Diana'	—	120:120	178:178	298:298	82:91	—	151:163	277:320	282:282	182:227	200:200	285:285
V. molle	—	—	—	240:240	—	—	149:149	279:320	260:281	182:227	199:199	287:287
V. lantana	—	104:122	180:180	244:244	—	—	149:149	316:327	—	182:227	193193	269:269
V. ellipticum	—	—	182:182	—	96:107	—	—	316:327	301:301	182:229	195:210	267:291
V. dentatum 'Blue Muffin'	208:208	—	155:172	—	105:105	—	—	235:235	237:237	123:182	140:146	199:207
V. utile	—	120:120	180:180	260:300	100:163	—	—	242:260	196:196	146:179	144:150	214:214
V. tinus 'Compactum'	—	—	184:184	—	163:255	—	—	235:251	248:260	110:146	144:155	198:207
V. lentago	—	120:120	161:182	244:244	114:168	191:191	151:151	235:253	264:264	146:182	148:156	—
V. cinnamomifolium	—	—	—	—	170:175	—	—	233:253	216:216	146:179	150:155	210:210
V. prunifolium	9797	120:120	184:184	244:244	89:89	191:191	147:147	237:251	279:279	146:182	156:156	—
V. lobophyllum	—	—	—	—	—	—	147:147	231:244	—	146:177	151:158	166:205
V. rufidulum	111:111	120:120	180:180	244:244	9191	217:217	153:153	235:253	2552:55	146:195	156:156	—
V. melanocarpum	—	—	—	—	159:159	—	153:153	253:253	183183	146:177	150:158	—
V. nudum	111:111	—	182:182	244:244	—	—	111:151	244:261	200:209	148:180	142:148	198:210
V. japonicum 'Variegatum'	—	—	170:180	—	193:193	119:119	111:151	235:235	—	146:177	150:150	198:198
V. foetidum	—	—	155:172	—	105:105	—	—	235:235	—	146:177	150:153	166:207
V. propinquum	97:159	—	180:180	260:300	100163	—	—	206:251	210:210	150:177	130:144	144:185
V. luzonicum	—	—	184:184	—	163:255	—	—	233:250	266:266	146:177	148:160	189:194
V. bracteatum 'Emerald Luster'	—	120:120	161:182	244:244	114:168	191:191	151:151	235:251	—	130:179	146:164	—
V. farreri 'Nanum'	—	—	—	—	170:175	—	—	253:253	181:181	179:179	104:150	212:241

Table 4-2. Continued.

Allele sizes (bp) for loci

Accession	VR005	VR008	VR011	VR012	VR028	VR043	VR044	VF001	VF002	VF004	VF005	VF007
V. rhytidophyllum 'Cree'	97:97	120:120	180:180	244:244	91:91	217:217	153:153	239:239	191:191	146:179	146:146	208:208
V. phlebotricum	—	—	—	—	—	—	147:147	237:251	264:264	146:177	156:199	—
V. awabuki 'Chindo'	111:111	120:120	180:180	244:244	91:91	217:217	153:153	255:255	205:205	151:182	146:146	208:232
V. grandiflorum	—	—	—	252:252	1591:59	—	153:153	253:253	192:192	180:180	153:153	212:227
V. ichangense	111:111	122:122	182:182	244:244	—	—	111:145	242:242	—	146:177	150:155	167:199
V. bitchiuense	—	—	170:180	—	193:193	119:119	111:151	237:237	207:207	146:179	144:153	207:207
V. burejaeticum	—	104:122	180:180	248:300	103:103	—	154:154	248:248	200:200	145:177	146:151	207:212
V. dasyantum	—	—	155:155	—	82:82	—	154:154	233:233	—	121:177	138:164	198:207
V. recognitum	—	—	—	—	—	—	—	207:244	229:229	146:175	146:158	208:208
V. wilsonii	—	—	—	—	139:139	161:161	153:153	239:239	—	143:173	146:146	196:201
V. acerifolium	—	—	176:176	—	163:181	117:117	—	239:250	237:237	145:179	133:133	—
V. dilatatum 'Asian Beauty'	—	—	176:176	—	163:184	—	—	250:250	262:262	145:177	140:140	—
V. erosum	—	156:156	—	—	—	—	—	237:237	240:240	145:175	128:140	—
V. davidii	—	—	—	—	161:172	—	—	229:229	202:216	145:175	128:140	201:201
Adoxa moschatellina	—	—	—	—	138:138	—	—	—	—	177:177	—	—
Sambucus canadensis	—	—	100:100	—	—	—	—	—	237:237	175:175	—	—
V. atrocyaneum	104:104	136:145	174:174	—	87:195	—	—	229:246	233:233	168:177	88:153	201:201
V. buddleifolium	—	134:145	—	—	103:103	—	—	237:250	189:202	146:177	130:137	207:207
V. hupehense	131:131	134:143	145:145	—	161:161	—	—	235:244	—	145:177	130:137	192:192
V. nervosum	—	122:122	178:178	—	103:103	—	—	237:237	185:209	146:177	130:130	207:207
V. opulous	171:171	134:134	112:112	—	159:181	—	—	237:237	181:187	146:179	91:130	192:192
V. schensianum	—	104:122	182:182	—	—	—	—	239:274	187:187	145:177	124:135	207:207
V. setigerum	—	134:145	204:204	—	166:166	—	—	206:206	246:246	146:175	133:140	—
V. suspensum	—	134:142	176:184	—	112:179	—	—	250:250	187:187	168:180	140:155	229:236
V. cylindricum	—	134:142	176:184	—	112:179	—	—	235:235	222:227	168:180	140:155	229:236
Lonicera japonica	—	—	—	—	—	—	153:153	—	—	177:177	—	—
Weigela florida	—	—	—	—	—	—	151:151	—	—	—	—	—
Sambucus nigra	—	—	—	—	—	—	—	—	212:233	174:174	—	—

Table 4-2. Continued.

	Allele sizes (bp) for loci								
Accession	VF008	VF009	VF013	VF014	VF016	VF025	VF027	VF029	VF030
V. erubescens	179:179	247:247	182:182	135:139	146:169	136:148	206:214	181:181	173:173
V. odoratissum	181:181	—	182:182	135:187	146:171	148:148	206:206	183:183	173:173
V. trilobum	—	—	182:182	—	—	—	—	—	173:173
V. sieboldii	181:181	247:247	128:182	135:184	146:171	134:134	206:225	149:183	173:173
V. sargentii	181:181	243:243	180:180	132:162	143:180	130:134	198:198	147:179	173:173
V. plicatum	183:183	246:246	182:182	135:184	169:178		206:233	183:183	173:173
V. rafinesquianum	181:181	243:243	107:180	162:184	133:165	127:127	207:207	147:179	173:173
V. carlesii 'Diana'	181:181	243:243	182:182	189:189	133:167	146:154	215:219	147:178	171:171
V. molle	181:181	243:243	107:180	128:155	133:165	129:129	204:213	147:178	173:173
V. lantana	183:183	246:331	186:186	187:187	133:167	—	228:228	147:178	—
V. ellipticum	181:181	246:246	180:180	164:169	135:167	130:130	206:211	147:179	173:173
V. dentatum 'Blue Muffin'	139:139	236:236	180:180	168:186	133:163	130:130	217:217	149:183	173:173
V. utile	103:132	236:236	180:180	187:187	133:165	141:141	228:247	147:179	171:171
V. tinus 'Compactum'	—	234:280	182:182	146:146	96:113	130:130	211:211	110:147	173:173
V. lentago	128:128	234:234	180:180	175:187	131:169	148:148	234:234	147:183	173:173
V. cinnamomifolium	—	234:234	109:182	146:160	113:113	130:130	—	150:181	173:173
V. prunifolium	141141	234:234	176:176	162:182	131:169	136:159	232:240	147:183	173:173
V. lobophyllum	137:137	234:234	107:180	160:160	133:133	130:130	221:221	149:178	173:173
V. rufidulum	128:128	234:234	178:178	166:184	131:169	143:143	232:242	147:183	173:173
V. melanocarpum	137:137	234:234	142:180	164:186	133:165	130:130	225:225	149:178	173:173
V. nudum	128:128	234:234	180:180	186:186	133:167	150:150	—	149:181	173:173
V. japonicum 'Variegatum'	132:132	234:234	—	—	133:133	130:130	223:223	149:178	173:173
V. foetidum	—	234:234	186:186	164:164	133:165	129:129	209:209	147:178	169:169
V. propinquum	—	236:236	180:180	148:162	113:113	129:129	230:249	150:179	—
V. luzonicum	—	236:236	182:182	189:189	133:167	129:129	211:238	147:178	167:167
V. bracteatum 'Emerald Luster'	—	—	182:182	169:189	109:133	129:129	206:206	123:178	167:167
V. farreri 'Nanum'	—	236:236	182:182	196:196	167:167	168:168	206:219	179:179	167:167

Table 4-2. Continued.

Allele sizes (bp) for loci

Accession	VF008	VF009	VF013	VF014	VF016	VF025	VF027	VF029	VF030
V. rhytidophyllum 'Cree'	—	236:236	180:180	187:187	133:165	152:152	226:226	147:179	167:167
V. phlebotricum	—	236:234	109:180	160:187	133:163	129:129	213:219	147:176	167:167
V. awabuki 'Chindo'	—	—	126:182	133:193	146:171	130:143	209:226	183:183	167:167
V. grandiflorum	—	—	126:182	189:189	167:167	132:147	219:242	181:181	167:167
V. ichangense	—	236:236	105:180	159:159	133:133	129:129	213:232	147:176	167:167
V. bitchiuense	—	236:236	148:182	191:191	133:167	148148	217:223	149:181	169:169
V. burejaeticum	—	243:231	186:186	200:200	133:167	161:161	206:206	147:179	171:171
V. dasyantum	—	243:243	182:182	180:180	109:133	129:129	206:206	123:178	171:171
V. recognitum	—	243:243	182:182	180:180	109:133	129:129	206:213	123:178	171:171
V. wilsonii	—	243:243	—	173:173	133:141	129:129	108:207	145:176	171:171
V. acerifolium	126:126	236:236	182:182	166:173	133:167	129:129	209:209	145:145	—
V. dilatatum 'Asian Beauty'	124:124	234:234	182:182	162:187	133:143	130:130	209:209	145:176	171:171
V. erosum	234:234	—	142:180	162:186	133:143	129:129	—	—	—
V. davidii	—	234:234	109:109	148:193	113:113	130:130	—	149:149	—
Adoxa moschatellina	—	242:242	178:178	—	156:156	—	—	179:179	—
Sambucus canadensis	—	—	—	132:171	—	—	—	145:145	—
V. atrocyaneum	—	236:236	182:182	160:187	113:113	129:129	194:194	170:178	171:171
V. buddleifolium	—	322:322	180:180	186:186	133:165	141:154	225:225	147:178	171:171
V. hupehense	—	232:232	180:180	162:186	133:143	129:129	209:209	147:178	171:171
V. nervosum	—	236:236	184:184	187:187	133:167	157:157	225:225	147:178	169:169
V. opulous	—	234:234	184:184	160:187	135:145	130:130	200:200	—	171:171
V. schensianum	—	236:319	—	187:187	133:167	168:168	225:225	147:178	171:171
V. setigerum	—	234:234	180:180	164:186	133:143	129:129	209:209	147:178	169:169
V. suspensum	—	—	182:182	186:186	154:169	150:150	201:234	—	171:171
V. cylindricum	—	240:240	180:180	162:162	135:135	129:129	185:206	147:178	—
Lonicera japonica	—	—	—	227:227	133:167	—	—	178:178	—
Weigela florida	—	—	—	—	171:171	—	—	147:183	—
Sambucus nigra	—	—	—	160:160	—	—	—	123:145	—

Table 4-3. Characteristics of 33 SSR loci developed from *V. dilatatum*, *V. rufidulum* and *V. farreri* used to amplify *Viburnum* species and other closely related genera, including: primer sequence, repeat, number of alleles, number of amplifications, expected size, and polymorphic information content (PIC). V = *Viburnum*, A = *Adoxa*, L = *Loricera*, S = *Sambucus*, and W = *Weigela*.

SSR Locus	Primer Sequence (5′-3′)	Repeat	Number Of alleles	# of species amplications/genus					Expected size bp	PIC
				V	A	L	S	W		
VD003	F: TGGCTCAGATGCATTGAAGAATAG R: GCTGCATGCATCTTCAAATAGG	$(CA)_{12}$	26	42	—	—	—	—	143	0.93
VD004	F: GCTGCATGCATCTTCAAATAGG R: ATATCTCGAGGGAGACTGCAACAG	$(AC)_{16}$	18	33	—	—	—	—	156	0.87
VD005	F: TTTTAAAACTTTGCACCCTTGCAC R: AGAATAAAGTCCAGCTCCCTGACC	$(CA)_{7}$	27	37	—	—	—	—	178	0.93
VD006	F: ATAACCATATGCGTGTGTATGTTGG R: GACGTTGCAGGAGCTTCTTATCTC	$(GT)_{8}$	4	12	—	—	—	—	141	0.42
VD009	F: GTTTGGGACATGTTCAGTTCTTCC R: AATGTCAGCAAATCAAAATCCAAAC	$(TG)_{12}$	17	28	—	—	—	—	163	0.90
VD012	F: TCGACTCTACATTCACTACCCTCC R: CATACGGGTATACGCACACATGC	$(AC)_{16}$	8	9	—	—	—	—	174	0.80
VD014	F: GCAAACCAAACCACACAAACAC R: ATCTAGGTCGGCTGCTACTGATTG	$(CT)_{6}(CA)_{7}$	28	35	—	—	—	—	204	0.94
VD016	F: TACCCCTCACAAACACAAACACTG R: AACAATAATGGTGTGGGGTGTTG	$(AC)_{12}$	13	25	1	1	—	—	129	0.87
VD017	F: ACCAACCCAATTGCTCAATATCAC R: GGTTGTCCGCCAGAAGTAGTAGTG	$(AC)_{6}$	9	27	—	—	—	—	170	0.70

Table 4-3. Continued.

SSR Locus	Primer Sequence (5′-3′)	Repeat	Number Of alleles	# of species amplications/genus					Expected size bp	PIC
				V	A	L	S	W		
VD018	F: CTTGCTCGATTTCCCTTATTTGTC R: ATCTCAAGCAAGTCTCACTCCCTC	$(CA)_{16}$	6	35	—	—	—	—	112	0.42
VD019	F: AAAGTTGCAAATTACACGCTGATG R: TACCTCCAATTTCACGGTTCTCTC	$(TG)_{16}$	19	35	—	—	—	—	167	0.92
VR004	F:CTACCGCACATATGCACCTAC R:TACAGATCGGGAAGGTGTAAGG	$(TG)_6(TG)_7$ $(TGTGTG)_2$	14	23	—	—	—	—	210	0.91
VR005	F:TGCTTCCATCTCTTTTCTCTCC R:GTGTGTGGCTGTGTTTGTACG	$(TG)_6(TG)_7(GT)_7$	10	14	—	—	—	—	113	0.83
VR008	F:CCAAGCCCCATTTTATAAATACC R:CATTCTGCCATTTTATTGAGTCC	$(AAGAATTT)_2$ $(TTAAGAA)_2$	11	24	—	—	—	—	128	0.78
VR011	F:GCATATGTGCACACACGAGAG R:TATGGATTGGGAAGGTGTTAGG	$(CA)_{12}$	18	36	1	—	—	—	174	0.90
VR012	F:CAAAGTGGGCAGAGAAGTAGC R:CATTGTAGAGCACCACAAATTCC	$(TA)_7$	9	20	—	—	—	—	245	0.75
VR028	F:GCTCGTAGCAGGGGTGTGTAT R:CACGCACACTGCACACACT	$(CA)_5(AC)_8$	35	40	1	—	—	—	116	0.96
VR043	F:TATGTGAGGATGAAGGTGATGG R:TTACATTCTGGCAGTAGCAACC	$(CT)_7(TC)_5(TC)_5$	7	12	—	—	—	—	213	0.76

Table 4-3. Continued.

SSR Locus	Primer Sequence (5′-3′)	Repeat	Number Of alleles	# of species amplications/genus					Expected size bp	PIC
				V	A	L	S	W		
VR044	F: CTCTCTTCATCCATGCTTCTCC R: TGACGAAAATTGATGATTAAAACC	$(CT)_{14}$	9	27	—	1	—	1	165	0.75
VF001	F: GCACTTTGCAAAACCATACC R: ATTTGCTAGTGTACGTGTGC	$(CT)_{7}$	29	50	—	—	2	—	144	0.94
VF002	F: ATGATACTCCCAAGCATGATCC R: ACTCATTGTCTGTCTTGATGG	$(GT)_{3}$ $(GA)_{5}$	39	42	—	—	—	—	194	0.97
VF004	F: GTGGTTGAGTATTGCAGGAAG R: TCTACACACACCACACACAACC	$(GT)_{6}$	24	50	1	1	1	—	176	0.88
VF005	F: CCCAAATTGCTTATTCTTCTGC R: GCATTCTTTGAACCCTTTGG	$(TG)_{5}$	33	50	—	—	—	—	154	0.95
VF007	F: CAAACTTTTAGGGGTAATTAGG R: AGTGATGTAAATAGGCGGAAGC	$(GT)_{6}$	32	42	—	—	—	—	188	0.95
VF008	F: GCATAACCCTCGTCATAGGC R: TGGGTGTATAGTGTGCGTAGC	$(CA)_{12}$	12	22	—	—	—	—	200	0.83
VF009	F: TCCTAGAGGGGGTGAATAGG R: TTTCCCTTCTTGGGTTTTCC	$(GT)_{13}$	12	42	—	—	—	—	168	0.78
VF013	F: TTCTTTAGCTGCGAGTGTGTGT R: GGATTCGTCGATTTTGGTAAAG	$(GT)_{6}$	14	46	—	—	—	—	200	0.73

Table 4-3. Continued.

SSR Locus	Primer Sequence (5′-3′)	Repeat	Number Of alleles	# of species amplications/genus					Expected size bp	PIC
				V	A	L	S	W		
VF014	F: ACTGCACATATGCACACACG R: GGGACTAGATTACGGATTGG	$(CA)_4$	29	46	—	1	2	—	190	0.94
VF016	F: ACACACCACACACAACCACA R: AGTATTGCAGGAAGGCATGG	$(CA)_4$	20	48	1	1	—	1	164	0.85
VF025	F: CCTGTGCCATAAGAAGC R: GGTGAATATGGATAAGCAAAAGC	$(GT)_7$	18	46	—	—	—	—	160	0.80
VF027	F: TGACAACTCTGACCACGATAC R: AAGAGATTAGGGTTCTTAGTTTTGG	$(TA)_6$	18	43	—	—	—	—	240	0.80
VF029	F: GTGGTTGAGTATTGCAAAGG R: TCTACACACACCACACACAACC	$(GT)_{12}$	12	45	1	1	2	1	176	0.84
VF030	F: TTCCTTTCAATAACCTCAACATCC R: TCGGATGAAGTATATTTTTGG	$(TC)_4$	4	43	—	—	—	—	156	0.63

APPENDIX 6: FIGURES

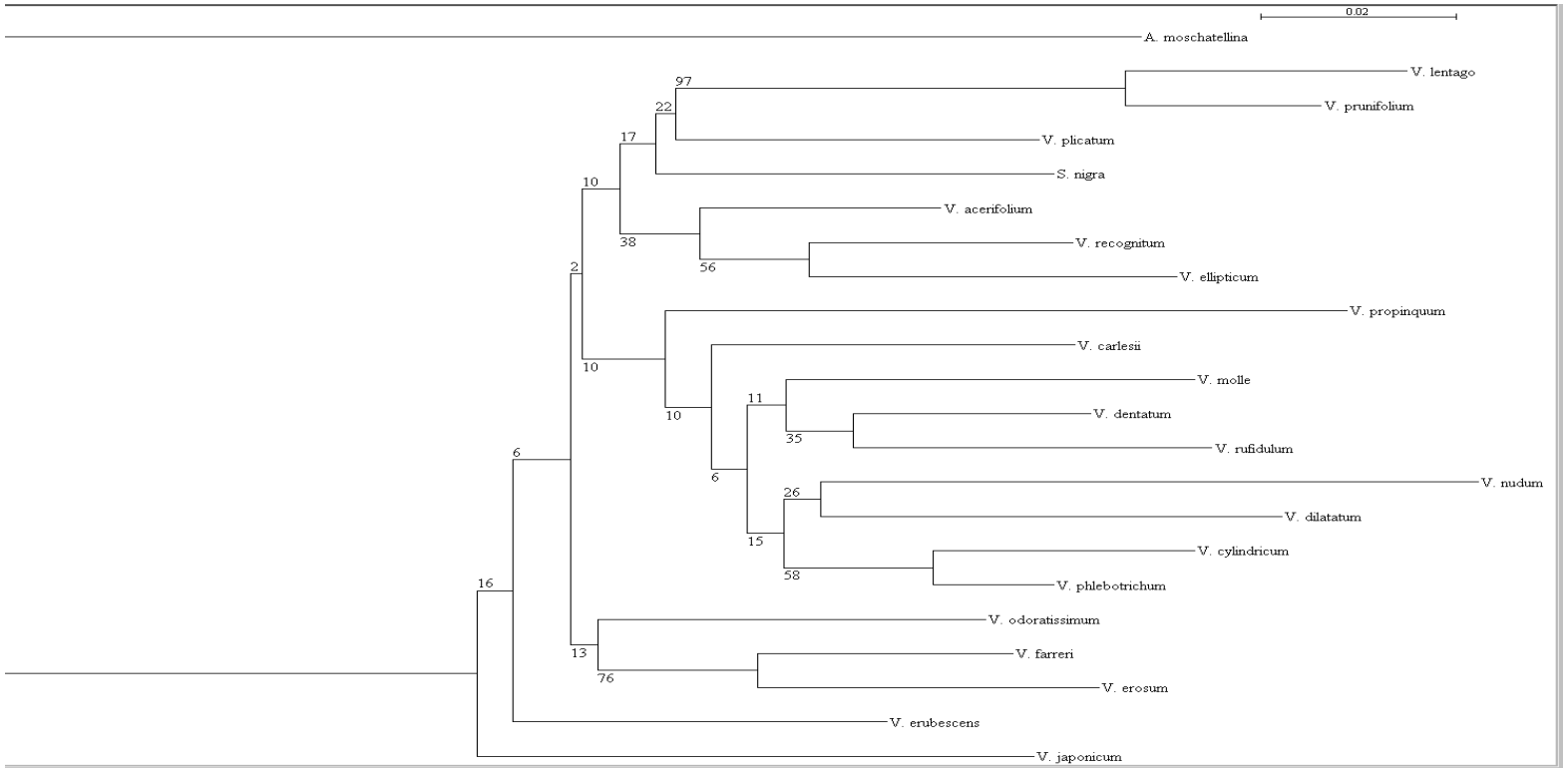

Figure 4-1. A neighbor joining rooted tree using cross transfer data from three *V. farreri* loci to analyze the relationships of *Viburnum* and the closely related genera: *Adoxa*, *Loricera*, *Sambucus*, and *Weigela*. The bootstrap values for 1,000 randomizations are shown at the right of each node. The data was generated and viewed using the program Seaview 4.5.2 (Gouy et al., 2010). The scale bar represents 0.2 substitutions per nucleotide site.

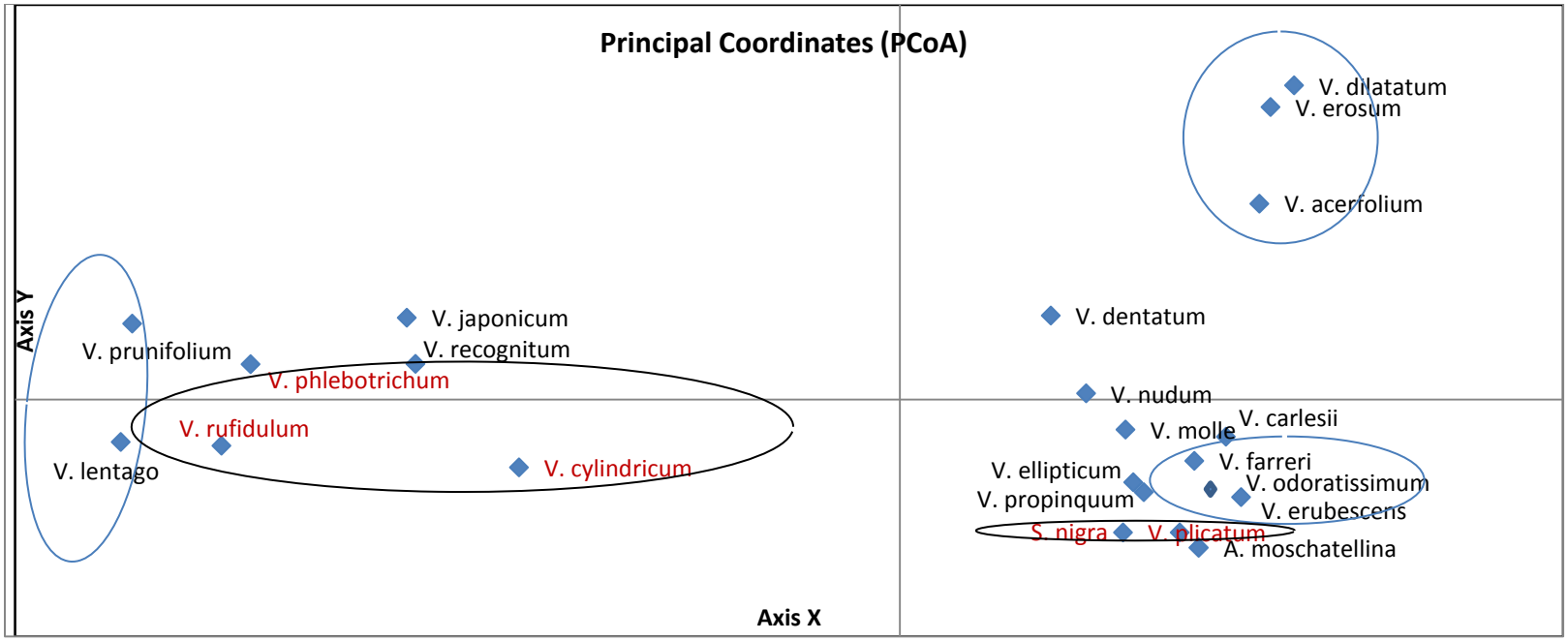

Figure 4-2. A principal coordinate analysis (PCoA) based on 33 *Viburnum* SSR loci and 22 accessions of related genera carried out in the program in GenAlEx version 6.5 (Peakall and Smouse, 2006). Blue circles show relations in agreement with Donoghue and Winkworth (2005), and black circles with red font show conformity with relatedness in this study.

Chapter 5. Conclusion

Concluding Remarks

In this dissertation three *Viburnum* SSR libraries were constructed to assess the genetic diversity of the genus *Viburnum*.

The *Viburnum dilatatum* microsatellite markers have potential for identifying *V. dilatatum* cultivars, and its hybrids. This set of microsatellite markers will be useful in marker-assisted selection, gene mapping, population genetics, and identification of *Viburnum* species with invasive potential. Two- to- twelve alleles were identified for each locus, and the polymorphism information content (PIC) values ranged from 0.36 to 0.87. The expected heterozygosity (He) ranged from 0.48 to 0.88 and observed heterozygosity (Ho) ranged from 0 to 0.73. This set of molecular markers also exhibited transferability between various *V. dilatatum* cultivars and hybrids with *V. japonicum*. As a consequence, these markers will aid in breeding for new cultivar development, assist with early detection and screening of plants that have escaped cultivation, and are expected to help in refining the phylogenetic relationship of *V. dilatatum* to other species and genera within the Adoxaceae.

The SSR library developed from the native species *V. rufidulum* were useful in conducting a population genetics study. The markers provided information concerning the range of genetic diversity present in native localities that were previously uncharacterized in the species, *V. rufidulum*. The structure of these populations of *V. rufidulum* can most likely be attributed to habitat fragmentation and the resulting geographical isolation. The similarity of the genetic clusters suggests that the populations are fragmented remnant populations that may have once been one large contiguous population of trees within the native range of the species. This study revealed many

informative alleles, making this set of SSR loci useful for identifying the levels of diversity within other *V. rufidulum* populations as well as other Viburnum species. In the future studies may benefit from using additional markers and incorporating more populations across a broader range of *V. rufidulum* and also including collection locales that are separated by geographical barriers. *Viburnum rufidulum* is an important native tree that has attractive ornamental qualities, and we expect this set of microsatellite markers to be of value to breeding programs that aim to utilize the attractive traits of the tree and to promote the use of native plants as an alternative to potentially invasive non-native species.

Finally a novel *V. farreri* SSR library was utilized to characterize 33 *Viburnum* loci and assess cross transferability in Adoxaceae and Capriloliaceae, and were used to attempt to construct a phylogeny of the genus *Viburnum*. The cross species transferability demonstrated by the loci may assist in guiding wide hybridization selections. Moreover, the high rate of conservation of primer sites suggest that this set of markers will be of utility in further intra and interspecific studies. The resolution of the phylogeny of the genus *Viburnum* with SSR markers could benefit by including many more makers, and SSRs that occur in coding regions as well sampling more than one individual representative of each species. The limited number of markers used in this study and the lack of congruence found in the individual trees may have reduced the ability to resolve the concatenated data tree. This set of *Viburnum* markers did exhibit cross transferability to other genera and many did amplify in other *Viburnum* species. This set of loci did not provide resolution of the phylogenetic tree, but we expect the markers to be of utility for

breeding programs that wish to make wide hybridizations and species and cultivar identification.

Vita

Deborah Dean was born in Baltimore, Maryland. After serving in the United States Army she completed a B.S.N. from the University of Maryland at Baltimore. Deborah then received a Ph.D. from the University of Tennessee, Knoxville, TN in 2014 in Plants, Soils, and Insects with a concentration in plant breeding and genetics. She was mentored and advised by Dr. Robert N. Trigiano